



**Effects of Cadmium on Neutrophil Biology and
the Potential Role in Autoantigen Formation in
Rheumatoid Arthritis**

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Abstract

Rheumatoid arthritis (RA) is an autoimmune disorder for which there is no known cause. The disease is characterized by progressive joint destruction, mediated by breakdown of immune tolerance by immune system dysfunction, influenced by several factors. This results in the development of self-reactive proteins, called autoantigens. In RA, the dominant autoantibodies are directed to modified host proteins that are citrullinated and carbamylated. These modifications are driven by peptidyl-arginine deiminase (PAD) and myeloperoxidase (MPO), which are found in the greatest quantity in neutrophils, the major white blood cell of the immune system. The primary anti-microbial activity of these cells is characterized by several distinct functions: phagocytosis, degranulation and neutrophil extracellular trap (NET) production. NETosis is a unique cell death mechanism in which neutrophils unwind chromatin fibres from histone proteins, and through packaging with antimicrobial peptides, are released from the cell to ensnare and kill bacteria. These cells have been previously suggested as a driver in RA pathogenesis. The toxic heavy metal, cadmium, has been suggested in recent years as a potential contributing factor in RA, although the effect of cadmium exposure on neutrophil biology and function has not been fully explored. This thesis aimed to investigate serum autoantigens in RA, and explore the potential interplay between cadmium and neutrophil function in order to discern any dysfunctional processes that could promote autoantigen development.

Primary neutrophils were incubated with cadmium chloride and the effect on essential cellular functions was examined. Cadmium did not modify the phagocytic capacity of neutrophils, although it increased intracellular calcium

levels. Cadmium also enhanced the processes of the reactive oxygen species production and NETosis upon co-stimulation with phorbol 12-myristate 13-acetate (PMA) - an activator of protein kinase C.

Serum samples from 10 healthy control donors, 18 patients with RA, 4 patients with bronchiectasis (BR) and 4 patients with bronchiectasis and RA (BRRA) were analysed by western blotting to identify citrullinated proteins. Bands of interest were excised and identified using mass spectrometry. The heavy chain of immunoglobulin G, in addition to alpha-1-antitrypsin were found to be citrullinated in all samples, though in a much greater extent in RA patients. In addition, the same proteins also had a greater level of carbamylation than citrullination.

Taken together, these modifications provide novel evidence into how post-translational modifications may play a role in rheumatoid factor development in RA patients. As both PAD and MPO require elevations in the cytosolic calcium level to function, the effect of cadmium on neutrophil function may play a priming role in the cell, such that a second stimulus may give rise to a more exaggerated response during inflammation and a more permissive environment for autoantigen development.

Future work should examine the effect on MPO and PAD release after a period of cell priming with cadmium, and direct effects on immunoglobulin carbamylation/citrullination. This may allow greater insight into potential pathways through which RA may develop.

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Abbreviations and definitions

A1AT	α -1 Antitrypsin
A1ATD	α -1 Antitrypsin deficiency
ACPA	Anti-citrullinated protein antibodies
Anti-CarP	Antibodies to carbamylated protein
Anti-CCP	Antibodies to cyclical citrullinated peptide
(Anti-) _{cit} CRT	(Antibodies to) citrullinated calreticulin
BALT	Bronchial Associated Lymphoid Tissue
BCR	B cell receptor
BR	Bronchiectasis
BRRA	Rheumatoid Arthritis patients with Bronchiectasis
BSA	Bovine Serum Albumin
CD	Cluster of differentiation marker
Cd	Cadmium
CF	Cystic Fibrosis
COPD	Chronic Obstructive Pulmonary Disease
CRP	C-reactive protein
CTL	Cytotoxic T Lymphocyte
DHR123	DiHydroRhodamine 123
HBSS	Hank's Buffered Salt Solution
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgH	Immunoglobulin Heavy Chain
IgGHC	Immunoglobulin G Heavy Chain
IgM	Immunoglobulin M
IL1 β	Interleukin-1 β
IL-8	Interleukin-8
ITAM	Immunoreceptor Tyrosine-based Activation Motif
LPS	Lipopolysaccharide
MFI	Median Fluorescence Intensity
MHCI/II	Major Histocompatibility Complex class 1 or 2
MMP9	Matrix Metalloproteinase 9
MPO	Myeloperoxidase
MT	Metallothionein
NE	Neutrophil Elastase
NET	Neutrophil Extracellular Trap
NETosis	A form of cell death resulting in extracellular DNA release
PAD	Peptidyl-arginine deiminase
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0.1% Tween-20

PKC
PMA
PSM
RA
RA_{any}

RCF
RF
RFU
ROS
TCR
Th1/2/17
TLR
TNF- α

Protein Kinase C
Phorbol 12-myristate 13-acetate
Peptide Spectrum Match
Rheumatoid Arthritis
A cohort of individuals composed of
patients with both Rheumatoid
Arthritis and Rheumatoid Arthritis with
Bronchiectasis
Relative Centrifugal Force
Rheumatoid Factor
Relative Fluorescence Units
Reactive oxygen species
T cell receptor
T helper cell subtype 1, 2 and 17
Toll-like receptor
Tumour Necrosis Factor- α

1 Introduction

1.1 Cadmium and the effects of exposure on human physiology

Cadmium is highly toxic to both humans and animals alike, with several avenues of exposure in the general population. By exploring the bioavailability of cadmium once ingested into the body, it is possible to hypothesise how long-term exposure to cadmium may lead to cumulative cellular dysfunction, potentially resulting in chronic disease development.

1.1.1 Uses of cadmium in industrial applications and routes of occupational exposure

Once harvested from the earth, the aforementioned characteristics allow it to fill a niche role that other metals may not. The primary usage for cadmium, accounting for 86% of all cadmium globally used, is in the manufacturing of nickel-cadmium rechargeable batteries (1). An emerging use in recent years for cadmium is its application in solar-powered technologies. Cadmium-Tellurium batteries can convert sunlight into electricity at a higher rate, lower carbon footprint, and ultimately, lower cost than conventional silicon solar cells. This poses a potential health implication in the future given the current expansion of the solar technology industry.

Additionally, 9% of cadmium used globally is used in the process of colour pigmentation. It is added to plastics or paints, in order to make the red, yellow and orange shades look more vibrant. In recent years, this has prompted discussion to consider banning the use of cadmium in this practice, though ultimately it was allowed to continue. The final and less-common application for cadmium is to use as a coating for alloys and metals to protect them from corrosion (1).

Because of the manufacturing processes that the above products undergo, the individuals most commonly exposed to cadmium are typically employed in the metalworking industry. Metal workers inhale cadmium particles from dust/fumes from the processes of smelting, grinding and welding, where metals or alloys coated in cadmium particles are shed during processing where they may be subsequently inhaled (1, 2).

1.1.2 Non-occupational routes of cadmium exposure

The main sources of cadmium exposure for the general population are from food products and smoking. In food products, agricultural use of phosphate fertilizers containing cadmium, result in trace quantities to be present in food. In addition to this, run-off from industrial processes causes dispersal into the water systems and nearby soil, resulting in low levels of cadmium to be present in the environment. It is a consequence of this that cadmium is taken up rather avidly in certain plants and animals compared to other metal contaminants such as lead and mercury (3). This bio-accumulation of cadmium in plants crosses to organisms higher up in the food chain, whereby feeding on plants and animals containing high levels of cadmium can lead to long term detrimental health effects. The average person ingests roughly 30-50µg cadmium/day, though the amount ultimately accumulated can vary from 6-44% of the original dose, depending on age, though the authors of this study could not explain why this was the case (4).

For the general population, the largest and most abundant route to be exposed to cadmium is through smoking. The tobacco plant species (*Nicotiana spp.*) has a genetic predisposition to cadmium accumulation compared to other plants (5).

As this cannot be removed in the manufacture of cigarettes, it is passed on to smokers through the means of inhalation. Cigarettes from popular brands contain as much as 2µg of cadmium per cigarette, with roughly 10% of this transferring to inhaled cigarette smoke (6, 7). Considering the average person over 16 in the UK smokes roughly 11 cigarettes per day, as much as 2.2 µg of Cadmium could be inhaled by an individual on a daily basis (8).

1.1.3 Human response to cadmium exposure

The primary response to cadmium exposure is mediated by metallothioneins (MT). MTs are a family of proteins where the primary function is to bind mono and divalent metal ions, in order to maintain homeostasis of essential ions such as zinc or copper in the blood (9-11). MTs are also important in scavenging free radicals, providing protection against oxidative stress (12). They achieve this function through a high content of cysteine, which readily binds free metal ions or reactive oxygen species. Binding of MT to cadmium somewhat mitigates the toxic effects (13). The genes coding for production of MTs are found in all cell types throughout the body (particularly hepatocytes,) becoming activated upon exposure to metals (14). It is important to note that these levels in cells are very minute, and do not prevent cadmium from circulating in the body.

Upon inhalation of cadmium, between 10-40% of inhaled cadmium is retained in the lungs up to 94 days, though different sources of cadmium appear to result in differing levels of deposition (15). Cadmium chloride appears to be retained in the lungs at roughly twice the amount of cadmium sulfide. This could be due to the routes of administration, as CdCl₂ was administered via aerosol compared to the dose of CdS, which was given via dust particles, suggesting a droplet

containing cadmium may pose a greater risk than dust during inhalation (15). High abundance of Cadmium in the lungs can have negative consequences, which will be discussed later in Chapter Three. The entrance of cadmium into the body is hypothesised to be as a result of “ionic mimicry” (16). Ionic mimicry is a concept where cadmium ions act as homologues for other elements that may be transported across epithelial cell membranes (16). From here it can subsequently cross the pulmonary capillary bed and enter the circulation.

Once cadmium enters the bloodstream, cadmium binds to albumin, haemoglobin, cysteine residues and glutathione and circulates in the body. These complexes eventually accumulate in the liver (17). Fortunately, the MT anti-oxidants are also prevalent in the liver (18). Here, these complexes induce production of additional MTs that form new complexes with cadmium in place of the aforementioned proteins as a result of greater binding affinity (18). Chronic low doses of cadmium have been found to increase levels of MT in the body, leading to a protective effect against the toxic effects of cadmium (19). However, as MT-cadmium-protein complexes accumulate, hepatotoxicity can occur in hepatocytes, resulting in additional release of stored cadmium-MT complexes into the local tissue environment and blood circulation (20). These complexes ultimately become deposited in the kidney via renal circulation. Cadmium-protein complexes are not easily internalised by cells, apart from cells in the proximal tubule of the kidney (21). This deposition occurs through glomerular filtration, whereby the cadmium becomes transported into the cortical and distal tubules, as a result of protein reabsorption (22). It is important to recognise that not all cadmium-protein complexes are replaced by cadmium-MT complexes, and some complexes, such as cadmium-cysteine or cadmium-glutathione are capable of being internalised by proximal tubule epithelial cells

(20). Storage of cadmium in the kidney can last between 14-25 years (23). A person who is routinely exposed to cadmium could accumulate a substantial amount of cadmium over time, resulting in substantial health implications. A summary of routes of exposure and the physiological response to cadmium exposure can be seen in **Figure 1.1**.

1.2 Toxic effects of cadmium exposure

The cytotoxic effects of cadmium have been well-documented (24-29). Given the long-lasting duration of cadmium retention in the body, cadmium can induce both acute and chronic toxicity.

Acute toxicity is defined by the International Union of Pure and Applied Chemistry as the adverse effects that occur within two weeks after exposure to a single or multiple dose within 24 hours of a starting point (30). Chronic toxicity covers any symptoms observed after this time frame.

1.2.1 Oxidative stress

Oxidative stress plays a role in cadmium toxicity. As previously stated, cadmium bio-accumulation in hepatocytes induces production of MTs, through free Cd^{2+} ions entering the mitochondria and triggering oxidative stress (31). Here, Cadmium is capable of inhibiting the electron transport chain through impairing the function of cytochrome bc₁, driving generation of reactive oxygen species (ROS) (31). Cadmium abundantly binds intracellular glutathione and indirectly contributes to further ROS production (20, 32). These ROS are then able to induce DNA damage and apoptosis in the cells. This is evidenced by studies demonstrating apoptosis of renal and hepatic cells exposed to low

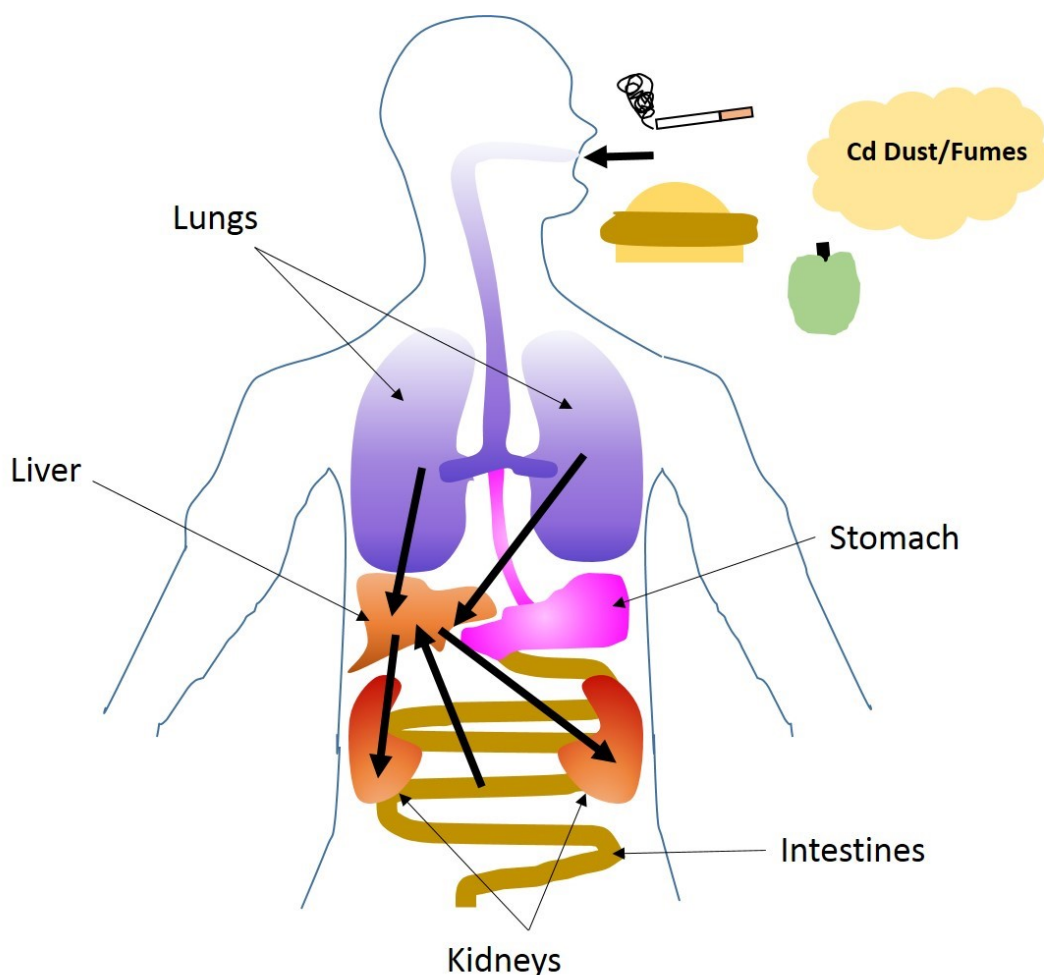


Figure 1-1: The routes of exposure and physiological human response to cadmium.

Smoking, food and workplace exposure are the primary routes of entry into the human body. Cadmium first arrives in the lungs and gastrointestinal tract based on route of exposure and enters the bloodstream. Here, cadmium forms complexes with various proteins in the blood and migrates towards the liver, increasing metallothionein production in order to sequester circulating cadmium and unbind it from the circulating proteins. These Cadmium-MT complexes then circulate to the kidneys, where they are absorbed by the proximal tubule component, and are retained in the kidneys for up to 25 years.

concentrations of 0.5 μ M of Cadmium over a 24h period, or 10 μ M leading to cell death within 6 hours. Higher doses of cadmium (50 μ M) were found to induce necrosis in mesangial cells (33). In the liver, ROS generation is further exacerbated by observations of cadmium initiating the Fenton reaction, another means of ROS production as shown in **Figure 1.2** (32).

1.2.2 Acute toxic effects

Ingestion of cadmium can cause vomiting of blood, severe abdominal pain, diarrhoea and myalgia (34). These symptoms can be brought on by as little as 3mg of cadmium in total, with a dose greater than 100mg being considered a lethal dose, with death resulting from cardiovascular collapse (35). Ingestion of such levels of cadmium over an acute period would be unlikely unless intentional. A more realistic form of acute toxicity would be through inhalation of cadmium.

Pulmonary cadmium toxicity is well documented in the form of chemical pneumonitis or metal fume fever (36-40). These conditions consist of thoracic pain and incredible difficulty breathing (36, 41). Although metal fume fever typically resolves due to a minimal extent of damage in the upper airways, chemical pneumonitis has a much more severe prognosis (34). Cadmium-induced chemical pneumonitis can lead to haemorrhagic bronchoalveolitis which has a mortality rate of 10-25% in cases (34). Both conditions are due to occupational exposure in areas of poor ventilation and lack of protective equipment. This presents little concern for modern industrial applications in western countries where health and safety measures are well maintained. Developing countries with less strict working conditions present the greatest risk for cadmium-related toxicity; with jewellery workers in India presenting a large group of individuals at risk (40).

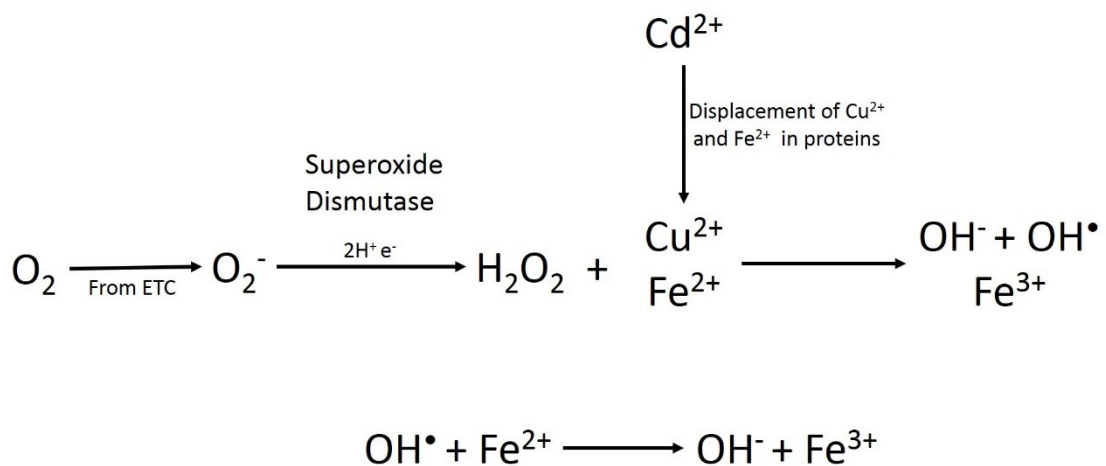


Figure 1-2 How cadmium contributes to the Fenton reaction.

Oxygen radicals are generated from *the* reaction between a free electron and oxygen. This radical is then converted to hydrogen peroxide through superoxide dismutase. The hydrogen peroxide then reacts with free copper and iron ions to form a hydroxyl radical. Cadmium releases copper and iron ions from cytosolic and membrane proteins such as ferritin through displacement. The other hydroxide with an unpaired electron then reacts with other iron ions to form an additional hydroxyl radical.

1.2.3 Chronic toxic effects

1.2.3.1 Pulmonary toxicity

The long-term effect of chronic cadmium exposure in the lungs should not be understated. Chronic obstructive pulmonary disease (COPD) and emphysema are the most common form of adverse events from cadmium exposure, with factory workers displaying the highest amount of cadmium exposure showing the greatest degree of lung abnormalities (42, 43). Interestingly, it has been proposed that it is in fact cadmium in cigarettes that is one of the driving factors in COPD and emphysema pathogenesis (37, 44).

1.2.3.2 Nephrotoxicity

The kidney represents the site of long-term cadmium deposition in the body, and subsequently is the primary organ affected by chronic cadmium toxicity. As previously mentioned, cadmium is capable of inducing cell death through oxidative stress (33). Tubular epithelial cells undergo apoptosis upon 24 hours of exposure to CdCl_2 , which was prevented by treatment with Propolis (an antioxidant product produced by bees,) evidencing the role of oxidative stress in renal cell death (45). Much of the damage to renal epithelial cells in the proximal tubule is facilitated by ionic Cd^{2+} as a result of breakdown of cadmium-MT complexes, rather than the complexes themselves (20) (28). Production of ROS induces cell death via DNA damage, though this damage also has another outcome: cancer.

1.2.3.3 Cancer

Cadmium is recognised by the World Health Organisation for Research on Cancer as a carcinogen. The most common cancer attributed to cadmium exposure is lung cancer (46-49). One study found that of 42 workers exposed to cadmium as part of their profession, four developed lung cancer - a hazard ratio of 3.23 (49). However, a recent meta-analysis of cohort studies examining the effect of cadmium exposure on lung cancer development found no statistically significant relationship to exist, but cited multiple reasons for the association to be underestimated (47). One of these reasons may be attributed to the “Healthy Worker Effect” whereby occupational studies can only recruit people who are currently healthy enough to work as opposed to severely ill people excluded from employment, potentially due to the variable in question (50). Most importantly, upon collating all the studies found into two groups of exposed and non-exposed individuals, there was found to be a statistically significant association between cadmium exposure and lung cancer. Given the highly toxic nature of cadmium in both acute and chronic cases, coupled with evidence of increased MT levels from repeated low-dose exposures, it is entirely possible for the severity of outcomes in occupational workers to be underrepresented (19).

The risk factor for renal cancer development is elevated in high-cadmium exposed individuals, in particular occupationally exposed individuals presenting the highest-risk group (51). Other cancers have been suggested to be in-part influenced by cadmium exposure, although there is insufficient evidence in human populations to verify any substantial risk (52, 53).

Further evidence of cadmium-induced carcinogenicity has shown that osteoblast cells exposed to 20 and 50µM of Cd for 24 and 48 hours caused

downregulation of checkpoint kinases and CDK2, whilst upregulating cyclin E2. Upregulation of cyclin E2 through upregulation of the CCNE2 gene is associated with cancer (54). The commonly used micronuclei assay as a means to detect genotoxicity in cells also found that both bone marrow and peripheral blood cells displayed higher levels of micronuclei in rats treated with 15mg/kg a day for 60 days (55).

1.2.3.4 Itai-Itai disease and associated bone defects

The first observed symptom of cadmium toxicity was in 1960's Japan, in the form of "Itai-Itai" (ouch-ouch) disease. A river was heavily polluted by mining slag, causing heavy metal pollution in the water, contaminating the crops with high levels of cadmium (56). This disease consists of renal dysfunction, severe bone pain and osteomalacia and osteopathy (57). Cadmium affects bone mineral metabolism in rats given a continuous dose of cadmium in drinking water, with calcium uptake in the intestines being negatively affected compared to untreated rats (58).

1.2.3.4.1 Reproductive dysfunction

Cadmium is known to interfere with hormonal functioning through binding both the nuclear and membrane bound receptor for oestrogen (59). In animal models, cadmium exposure elicits several detrimental effects to reproductive function (60). There is evidence to show testicular necrosis, failure of spermatozoa maturation, failure of oocyte maturation, and failure of oocyte implantation (60). Interestingly cadmium is stored in the testis of rats treated subcutaneously with cadmium daily over 6 weeks, reducing numbers of

spermatocytes compared to untreated rats (61). Human sperm have statistically significantly reduced motility when treated with cadmium as well as playing a role in impaired embryonic development in fertilised embryos (62). This evidence has been used to suggest cadmium as a cause for male infertility in certain parts of the world where high levels of exposure are common (63, 64).

1.2.4 Concluding remarks

Taken together, it can be concluded that cadmium has a highly detrimental effect on the human body. Through both cytotoxic and genotoxic means, cadmium exposure can cause or contribute to diseases that require substantial healthcare intervention. Cadmium causes disease pathologies that have been typically associated with smoking, and given the high affinity of the tobacco plant for Cadmium sequestration, it poses the question: how many pathologies caused by smoking are due to cadmium exposure? (37, 65)

1.3 The immune system

The immune system is essential for protection against microbial threats that could pose a problem for general physiological functioning. It consists of a variety of cells derived from different sites in the body that elicit different antimicrobial functions based on cell type. Each cell releases cytokines: signalling molecules which elicit a variety of functions based on the cell to which they bind. The immune system is broadly subdivided into two components: the innate and adaptive immune system. The innate immune system represents an evolutionarily conserved system that recognises pathogens via general recognition receptors and subsequently attempts to kill them upon activation.

This component alone does not resolve all infections, as not all pathogens may be recognised and killed at a rate that exceeds the rate at which they multiply. Alongside the innate immune system, the adaptive immune system works concurrently behind the scenes to generate antibodies that bind to pathogens and aid the innate system in resolving the infection. Each immune cell expresses a variety of surface proteins, called Cluster of Differentiation markers (CD). Each subclass of cell has different levels of expression of these markers, which makes identification based on these rather simple. A summary of each immune cell and how its' lineage is derived can be seen in **Figure 1.3**.

1.3.1 Cells of the innate immune system

The innate immune system presents the first line of cell-mediated defence upon infection of the host body. These cells are capable of activating through receptors which recognise both pathogen associated molecular patterns and damage associated molecular patterns. This class of receptors are called toll-like receptors, which consist of ten members (TLR1-10) in humans. These receptors each recognise specific components (TLR4, for example, recognises lipopolysaccharide from gram-negative bacteria, whereas TLR2 recognises peptidoglycan found in gram-positive bacteria.) The variety of stimulatory antigens that activate this class of receptors is vast so that the cells can respond immediately to a plethora of stimuli and initiate an immune response as quickly as possible before substantial damage can be inflicted (66).

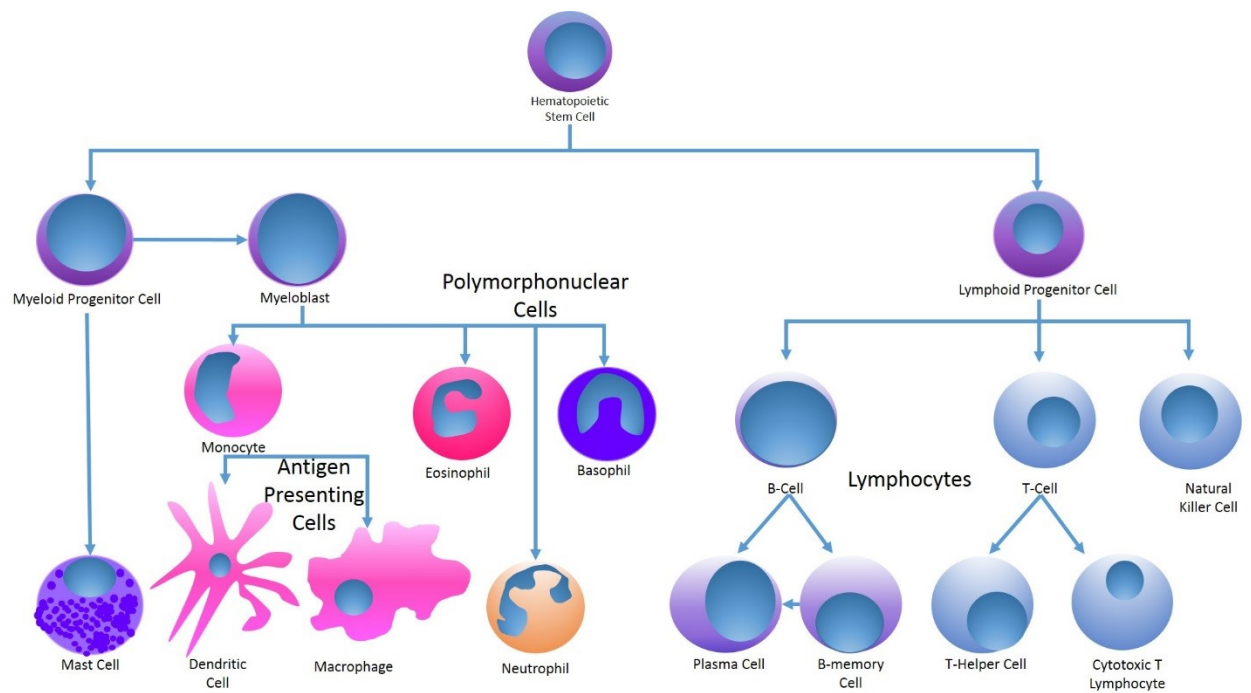


Figure 1-3: An overview of the lineages of hematopoietic stem cells and how each immune cell is generated.

1.3.1.1 Granulocytes

Granulocytes are a family of cells that are characterised by the presence of granules in their cytoplasm. Granules act as a repository for various cytokines and other immune mediators. The family of granulocytes includes four cells: basophils, mast cells, eosinophils and neutrophils.

1.3.2 Neutrophils

Neutrophils are the most abundant immune cell in the body. They are short lived cells, lasting roughly 16-24 hours in the body (67). Neutrophils circulate in the blood patrolling every aspect of the body and respond rapidly to threats. They are similar to macrophages in that both cells elicit their primary function through phagocytosis and the subsequent respiratory burst.

1.3.2.1 Life cycle

Neutrophils are derived from myeloid progenitor cells in the bone marrow at a rate of roughly 10^{11} cells per day (68). This can be even further increased during time of infection where cytokines influencing production are actively being produced. After production, neutrophils are stored in the bone marrow awaiting a stimulus in the form of a chemoattractant to induce release (68).

Once circulating, upon exposure to proinflammatory cytokines such as tumour necrosis factor- α , neutrophils become primed. Primed neutrophils are thought to have an enhanced capacity for functionality, with unprimed neutrophils possessing only a fraction of the antimicrobial potential (69, 70). This process is also thought to increase longevity of neutrophils, in order to prolong the activity of cells at sites of inflammation (70). Research suggests that neutrophils are

capable of de-priming upon resolution of inflammation through movement in the lung capillary bed (71, 72).

Once an infection has been resolved and stimulatory signals are no longer causing the cells to be activated, neutrophils enter an apoptotic state. During this state it upregulates the chemokine receptor CXCR4 so that there is increased responsiveness to CXCL12, a chemokine that directs neutrophils to migrate to the bone marrow or spleen in order to undergo apoptosis (73). Alternatively, apoptotic neutrophils may also remain at the site of infection and undergo cell death which is thought to exert anti-inflammatory effects which in turn switch macrophages into a phenotype that seeks to resolve inflammation (74). Once the process of neutrophil apoptosis has completed, the cellular remnants are then phagocytosed by the resident macrophages.

1.3.2.2 Phagocytosis

The process of phagocytosis is performed by neutrophils, macrophages and dendritic cells, and is outlined in **Figure 1.4**. As previously mentioned, innate immune cells have various pattern recognition receptors such as toll-like receptor-4, which recognises bacterial lipopolysaccharide however, in most cases, pathogen recognition is facilitated by opsonins such as immunoglobulins and complement proteins.

The receptors for immunoglobulin G on neutrophils are the FcγR family, receptors FcγRI-III. FcγRII (CD32) and FcγRIII (CD16) are constantly expressed on neutrophil surfaces, whereas FcγRI (CD64) is found on monocytes (75). FcRγI is only expressed on neutrophils after they have been activated, indicating signs of infection.

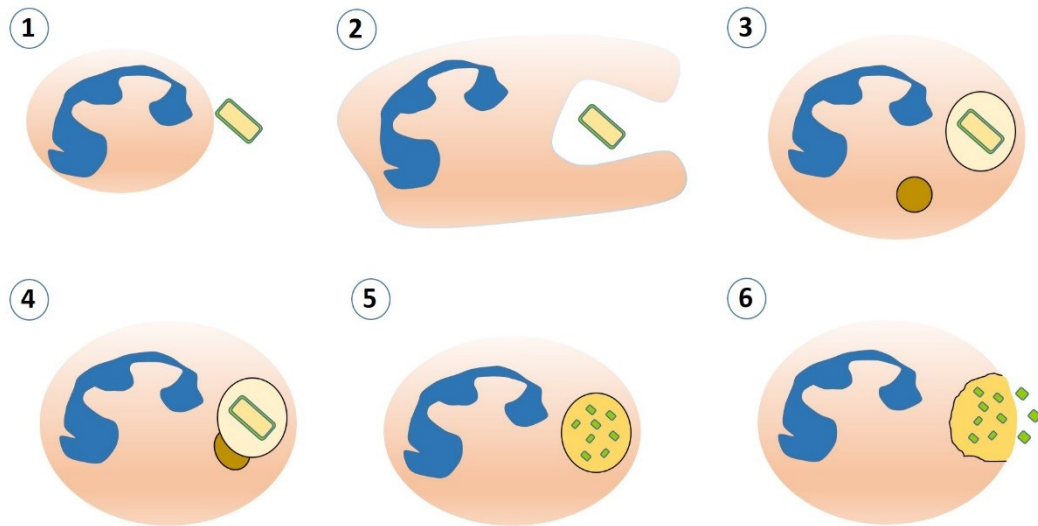


Figure 1-4 A summary of the process of phagocytosis.

1) The neutrophil encounters a pathogen and is activated through pattern recognition receptors, or opsonising agents. 2) The cytoskeleton surrounds the pathogen. 3) The pathogen is then encased in a membrane termed the phagosome. 4) Vesicles of highly antimicrobial compounds in the lysosome are trafficked towards the phagosome. 5) The fusion of the lysosome and phagosome forms the phagolysosome, and the antimicrobial compounds, in addition to production of reactive oxygen species result in pathogen lysis. 6) The contents of the phagolysosome are digested, and undigested components are released into the extracellular space.

The receptor for IgA, FcαR elicits a similar response to FcγR receptors, though IgA is unable to activate the classical pathway of the complement system, unlike IgG (75). Complement protein C3b binds to the CR3 receptor on neutrophils in order to trigger the same response as Igs. Toll-Like receptors (TLRs) may also be used for pathogen recognition, with each TLR recognising a particular form of pathogen associated molecular pattern.

In order for Ig-opsonised pathogens to initiate the process of pathogen engulfment, the antibody must activate both FcγRII and FcγRIII in neutrophils (76). These receptors contain immunoreceptor tyrosine-based activation motifs (ITAMs), which, when activated are phosphorylated by the Src-family kinases. Phosphorylated ITAM then activates the Syk-family kinases (77). The Syk-kinases in turn phosphorylate SLP-76, which recruits Vav and ADAP, resulting in actin cytoskeletal reorganisation, allowing the cell to engulf the pathogen (78).

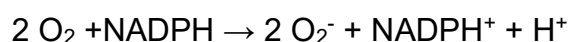
After engulfment of the pathogen, compartmentalisation occurs, forming the phagosome organelle. A lysosome containing digestive enzymes and ROS moves in the cytoplasm to fuse with the phagosome. Here, the phagolysosome forms and the contained organism is subjected to a variety of products that facilitate its lysis and degradation. The components of the phagolysosome that can be digested are absorbed by the neutrophil, and the components in the residual body that remain are released via exocytosis.

In the context of antigen presenting cells, rather than ejecting debris out of the cell, it may be processed into antigens and packaged with MHCII in order to present the organism components as antigens in order to facilitate antibody development. Lipid antigens such as mycobacterial cell wall components, may

also be presented by the CD1 marker, to provide an additional means of activating T-cells.

1.3.2.3 Respiratory burst

As stated in the process of phagocytosis, the predominant mechanism through which organisms are killed is via generation of reactive oxygen species, in a process termed the respiratory (or superoxide) burst. It is mediated by the enzyme NADPH oxidase, a multi-subunit protein complex. This complex is assembled upon cell activation, where phosphorylation-mediated activation of the cytosolic proteins, 40, 46 and 47 causes them to translocate to B cytochrome (p22 and p67 subunits)-containing membranes (79-81). Once the complex is assembled, the following reaction takes place:



This alone does not provide sufficient antimicrobial activity, though serves as a precursor molecule for several ROS (79, 82). The oxygen radicals are then paired with hydrogen H^+ ions to form the intermediate complex of hydrogen peroxide, H_2O_2 . This intermediate is then used as a substrate for the enzyme myeloperoxidase (MPO), which is used in generation of hypochlorous acid. Chloride ions are the most common substrate used in MPO-mediated reactions as Cl^- is highly concentrated in bodily fluids (83). Hypochlorous acid is a highly antimicrobial molecule that can create chloramines as an additional mechanism that may kill pathogens. Hydrogen peroxide may also be used as a substrate for the Fenton reaction. This reaction involves the oxidation of Fe^{2+} ions to produce hydroxyl radicals (84). These radicals are highly reactive and oxidise proteins of bacterial cell walls.

In the process of phagocytosis, the NADPH oxidase complex is formed on the membrane on the phagolysosome, resulting in the environment becoming a highly destructive and reactive zone through which pathogen lysis is achieved. The NADPH complex is also found on the membrane of phagocytes, allowing ROS to be released into the extracellular space in order to kill pathogens that have not yet been internalised (81). Unfortunately, the extracellular release presents a problem in that the ROS are then free to damage the surrounding area, which may kill local cells (83). Cells attempt to protect themselves from damage by upregulating cellular glutathione, though this is insufficient to protect the cells from apoptosis and necrosis if sufficient extracellular concentrations of ROS are produced (85).

1.3.2.4 Antimicrobial function through granule contents

In addition to phagocytosis and the respiratory burst, neutrophils contain potent antimicrobial proteins contained in the granules. Granules may be divided into several categories based on granular contents: primary, secondary and tertiary granules (86). Primary granules contain high levels of MPO, cathepsin G and various serine proteases (86). Secondary and tertiary granules, often referred to as peroxidase-negative granules, contain high levels of lactoferrin and matrix metalloproteinase-9 – MMP9 (also referred to as gelatinase B) though may exhibit a highly heterogeneous nature (87, 88).

Lactoferrin functions through chelation of Fe^{3+} ions produced through the Fenton reaction, so the free ions are unable to be metabolised by bacteria in order to reproduce (89). It also binds to bacterial membranes through binding to lipopolysaccharide (LPS), an abundantly expressed bacterial cell wall sugar

found in gram-negative bacteria (89). MMP9 is part of the matrix metalloproteinase family of enzymes that act on membrane proteins of cells or the extracellular matrix (90, 91). MMP9 is used to neutralise serine protease that may degrade the enzyme neutrophil elastase (NE).

Neutrophil elastase is an enzyme also stored in the primary granules of neutrophils. This enzyme works in tandem with ROS in the phagolysosome, though may be released additionally into the extracellular space during inflammation and infection. In the extracellular space, NE catalyses the breakdown of extracellular matrix components, in addition to structural proteins such as elastin, laminin and collagens (92). The remodelling of the extracellular matrix through NE activity aids the immune system in directing cells to the site of infection (93). It has also been found to kill bacteria through cleaving bacterial membrane proteins, as well as playing a large role in the formation of neutrophil extracellular traps (NETs) (94).

1.3.2.5 Antigen presenting cells

Antigen presenting cells circulate in the body, internalising molecules. Upon ingestion of pathogens, the bacterial debris is presented as an antigen on the MHCII. Macrophages, dendritic cells and B cells are all classed as antigen presenting cells. These cells Dendritic cells, in particular, act as a bridge linking innate and adaptive immunity. Following stimulation, and upon internalising a pathogen, dendritic cells travel to the lymph nodes to activate T cells and initiate the adaptive response, as outlined below.

1.3.3 Cells of the adaptive immune system

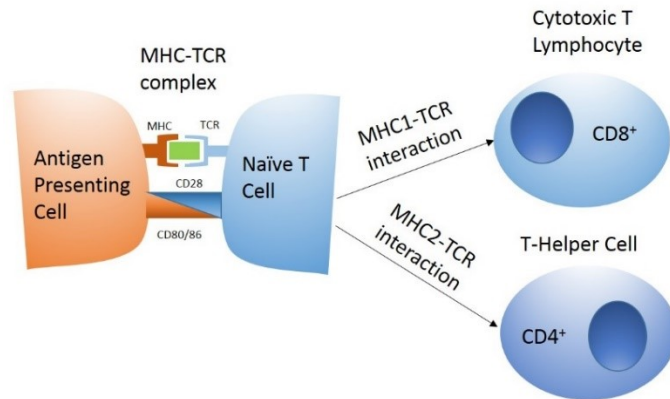
1.3.3.1 *T lymphocytes*

T lymphocytes develop in the thymus, though before they can leave the thymus and enter lymph nodes around the body, the T-cell is tested for binding affinity against self-proteins in order to prevent self-reactivity (95). Accompanying this, is expression of two key surface molecules, CD4 and CD8. The key defining characteristic of T-cells is the presence of the T-Cell Receptor (TCR) (96). This receptor is crucial to T-cell activation, and the interaction between the TCR and the major histocompatibility complex (MHC) determines the cell fate (**Figure 1.5**). When the TCR on T cells interacts with MHC class II, it sheds the CD8 surface marker and undergoes maturation and develops into a T-helper cell. Alternatively, if the TCR interacts with the MHC class I, the cell sheds the CD4 receptor, maturing into a cytotoxic T lymphocyte (CTL) (97).

T-helper cells can be further separated into predominantly two subtypes, Th1 and Th2 cells. Other variants of T helper cells include Th17 cells and T-regulatory cells. Th1 cells are principally involved in enhancing cell-mediated immunity, which involves enhancement of phagocyte and cytotoxic T-lymphocyte function. Contrastingly, Th2 are involved in stimulating antibody-producing cells and suppressing certain functions of phagocytic cells (98). These cells both share similarities in that without these cells, widespread activation of the immune system could not take place.

To dampen the immune response after resolution of bouts of inflammation, T-regulatory cells inhibit the function of T helper cells to prevent further stimulatory signals for the rest of the immune system. CTLs are involved in cell-killing functions.

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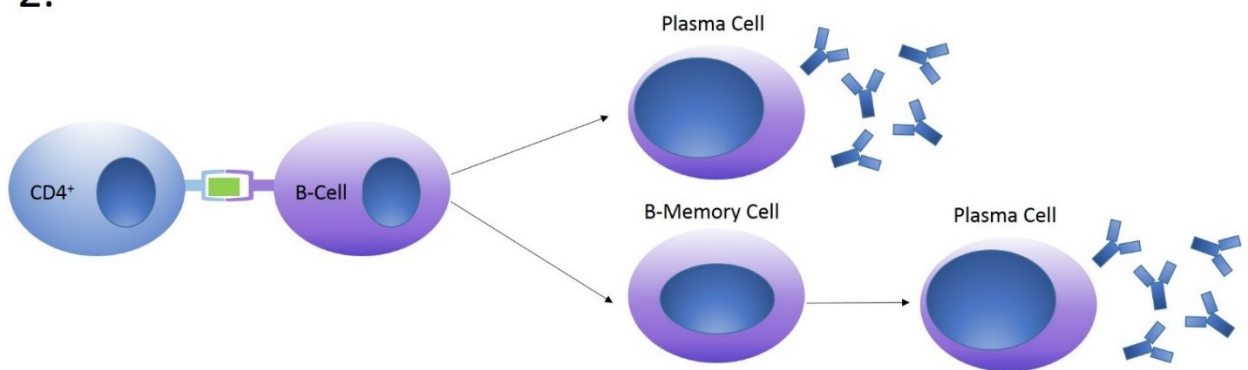


Figure 1-5 A summary of the process of T and B-cell activation.

1) Antigen presenting cells express the component of an antigen via the major histocompatibility complex surface protein. Naïve T-cells interact with this MHC with the T-cell receptor and activation occurs based on the MHC interaction. MHC I is expressed on all nucleated cells, and the MHC I-TCR complex induces generation of CD8⁺ cytotoxic T lymphocytes. MHC II is expressed on macrophages, dendritic cells and B-cells and the MHC II-TCR complex induces CD4⁺ T-helper cell generation. 2) T-helper cells present antigens via CD40-L to naïve B-cells via the CD40 marker and drive the generation of plasma cells that produce antibodies to the antigens. Upon successful generation of high-binding immunoglobulin, a B-memory cell is produced in addition to large quantities of plasma cells. B-memory cells provide a means to rapidly expand into antibody-releasing plasma cells upon re-exposure to the previously encountered antigen.

The majority of this killing is reserved to recognising and killing cancer cells, though in cases of viral infection, cells overexpress MHC I containing viral antigens, marking them for identification and death by CTLs (99).

1.3.3.2 B Lymphocytes

B-lymphocytes are generated in the bone marrow. Upon differentiation, B-cell fate can be predominantly B-memory cells, or plasma cells. The role of B-cells in the body centres on production of a crucial family of proteins: antibodies. This antigen is then internalised and displayed on the surface of the immature B-cell through the MHC II (100). Here, B cells attempt to synthesise antibodies greatest binding affinity for the antigen in question through somatic hypermutation (101). Once successful, the-B cell in question proliferates massively into plasma cells and enters the circulation.

A copy of the B-cell is retained in the form of a long-lived B-memory cell, retaining the BCR with the high affinity for the antigen, ensuring that upon encountering the antigen once more, a more rapid adaptive immune response occurs.

1.3.3.3 Antibodies

Antibodies are one of the most abundant family of proteins in the body. They are glycoproteins consisting of two heavy chains and two light chains linked by a S-S disulphide bridge (102).

Each immunoglobulin is composed of a constant and variable region (F_c and F_{ab}). The F_c region is responsible for interaction with complement proteins and docking with F_c receptors. The F_{ab} region is the antigen-binding region. An antibody molecule has two binding sites, given the Y-shaped structure of the

molecule. There are five classes of antibody: IgG, IgM, IgA, IgD and IgE. IgG is by far the most abundant class of antibody in serum (~12mg/ml), followed by IgA (~3mg/ml), IgM (~1.5mg/ml), and trace amounts of IgE and IgD (102). IgM is the first class of antibody produced in the early stages of an immune response due to the high avidity, though weak affinity for antigens.

IgG is a monomeric molecule with the greatest binding affinity for antigens, produced from immune system activation as described above. There are 4 subclasses of IgG (IgG1-4). IgG1 is the main subclass, produced in response to membrane proteins and soluble protein antigens (103). IgG2 is produced in response to bacterial polysaccharides, IgG3 is a potent proinflammatory antibody compared to other subtypes and IgG4 is produced in response to long term non-antigen stimuli (103). IgA is typically found in mucosal regions of the body, meaning it will be the first line of defence in most points of entry for the human body.

Immunoglobulins can perform multiple functions in the body. IgA is thought to cause aggregation of pathogens to allow mucous to trap them, in order to prevent further entry into the body (104). The action of antibodies binding surface antigens may also prevent entry of viruses and other pathogens by neutralising the proteins that facilitate infiltration into host cells (105).

Additionally, by binding the surface of the pathogen, complement activation through the classical pathway occurs, resulting in pathogen death through formation of the membrane attack complex (106). IgG and IgM are particularly strong activators of the complement system. Finally, and most commonly, antibodies act as opsonising agents. Opsonisation is the process of tagging an organism with multiple antibodies, to allow phagocytic cells to bind the Fc region

of an immunoglobulin and engulf the bound organism by the process of phagocytosis (107).

1.3.3.4 *Neutrophil extracellular traps*

Discovered in 2004 by Brinkmann and colleagues, NETs presented a novel death mechanism through which neutrophils were able to respond to pathogens (108). Neutrophils when exposed to immunostimulatory compounds were found to release fibres into the extracellular space that were composed of nuclear material that were able to ensnare and subsequently kill bacteria. In brief, the process of NETosis involves bonds between histone proteins in the nucleus becoming weakened, followed by chromatin relaxation. The unwound chromatin is then packaged with antimicrobial proteins in the cytosol, and then released from the nucleus into the extracellular space. It is activated by pathogenic stimuli such as LPS and is mediated by multiple enzymes. Since this discovery, NETosis has received considerable attention, with many questioning the literature regarding the authenticity of data, in addition to the mechanisms that underpin activation of the process (109-114). An overview of the process of NETosis, how it is activated, signalling pathways and the outcomes of the process are shown in **Figure 1.6**.

1.3.3.4.1 *Function of NETs*

Neutrophil extracellular traps are an antimicrobial mechanism that is exerted when a pathogen is resistant to phagocytosis due to size or resistance. NETs have been found to contain MPO and NE and defensins, providing a mechanism through which extracellular killing may be achieved.

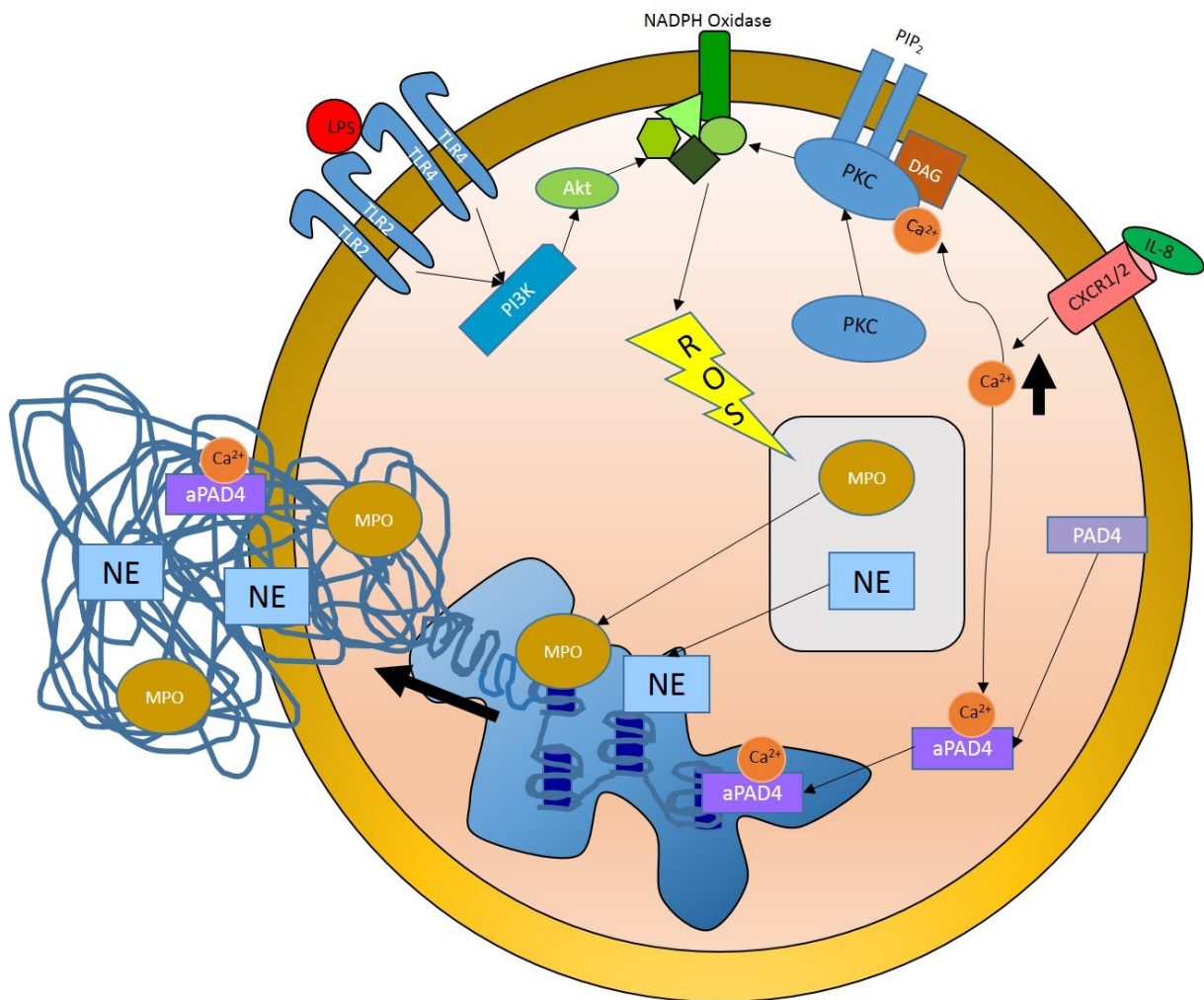


Figure 1-6 An overview of the process of NETosis.

NETosis can be induced through various molecules such as phorbol 12-myristate 13-acetate, lipopolysaccharide, heparin/platelet factor 4 antigenic complexes or interleukin-8 by various immune cells, generally neutrophils but also lymphocytes and macrophages. These molecules bind to their respective receptors and induce activation of PI3K/Akt pathways, protein kinase C activation, and increases in cytosolic calcium levels. These all result in formation of the NADPH oxidase complex, resulting in production of reactive oxygen species. This triggers degranulation and release of myeloperoxidase and neutrophil elastase into the cytoplasm. Increases in calcium level also lead to activation of cytosolic peptidylarginine deiminase-4. Here, MPO, NE and PAD translocate to the nucleus and weaken chromatin-histone bonds. These chromatin fibres are then repackaged with MPO, PAD and NE and released extracellularly, to aid in antimicrobial activity.

As DNA is highly sticky in nature, pathogens may be trapped in the web of DNA and subsequently killed by the proteins adsorbed to it (115).

1.3.3.4.2 NETosis induction pathways

In vitro, the only stimulus that consistently activates the process of NETosis is the protein kinase C activating, diacylglycerol mimetic Phorbol 12-myristate 13-acetate (PMA). NETosis has been observed in neutrophils treated with PMA concentrations as low as 4nM, though most research uses concentrations between 25-100nM (113, 116-119). This is most often used as a positive control for NETosis experiments when testing against other agonists.

Lipopolysaccharide and Interleukin-8 (IL-8) were the first biological agonists tested by Brinkmann and colleagues, initially suggesting this process as a means to degrade bacterial virulence factors (108). These results have been corroborated by other groups, suggesting that NETosis can be directly induced from not just bacterial extracellular stimuli, but also as a response to endothelial cell stress whereby IL-8 is released (120-122). Further research into cytokine-mediated NETosis reports that TNF- α and IL1 β are capable of inducing NETosis, though various levels of success are reported (113). Of most importance, is that cigarette smoke has recently been shown to induce NETosis, of which cadmium is a substantial contaminant (123).

1.3.3.4.3 Types of NETosis

Although NETosis was first observed as a death mechanism separate to apoptosis, autophagy or necrosis, it appears that this mechanism can be further stratified into different strands of outcome.

1.3.3.4.3.1 Suicidal NETosis

The classical form of NETosis has been dubbed “Suicidal” in that the fate of cells that undergo this process is death. Fuchs and colleagues confirmed Brinkmann’s findings of NETosis being a form of cell death by observing several steps before nuclear fibres were protruded out of the cell (121). After PMA stimulation, chromatin decondensation was preceded by nuclear envelope disintegration. This nuclear material was then found to be mixed with granule proteins in a vacuole-like organelle in the cytoplasm, before being expelled out of the cell (109). This process was found to take 3-4 hours before completion, suggesting cell death, given that bacterial-induced apoptosis in neutrophils has been observed to take roughly 2 hours (124). Yipp and Kubes make the point that suicidal NETosis seems counter-intuitive, as a mechanism that actively promotes cell death at the cost of cessation of other neutrophil antimicrobial activity seems redundant (109). They present a counterargument that when bacteria such as *S. aureus* produce toxins that can induce neutrophil cell death over a similar time frame, the process of suicidal NETosis may serve as a cellular kamikaze to ensnare bacteria that kill the NETing neutrophil, in the hope that another cell may be able to kill them (109).

1.3.3.4.3.2 Vital NETosis

Vital NETosis however, presents the idea of NET release without compromising the neutrophil’s ability to perform other antimicrobial functions. It has been suggested that NET-producing cells may be a subset of neutrophils, as neutrophils undergoing NETosis after having ingested bacteria would simply release the encapsulated bacterium back into the extracellular space. As NETs

are reported to occur in 20-25% of neutrophils, the hypothesis of this “NET positive” subset of cells may have some grounding (109).

Clark *et al.* found a population of neutrophils that released NETs, as confirmed by the extracellular, membrane impermeable dye SYTOX green when treated with LPS (125). Interestingly, the neutrophils did not uptake the dye, providing evidence that membrane integrity was not compromised in this instance. This is thought to be the first evidence of “Vital” NETosis. Of note is the rapid induction of this process (5 minutes), compared to the findings that the process is thought to take 3-4 hours.

Pilsczek and colleagues further corroborated this finding of NETosis induction upon treatment of neutrophils with *S. aureus* within a 60 minute window (126). This could be explained from the nature of which NETosis is induced. Suicidal NETosis has historically been observed from treatment of neutrophils with PMA. Given that PMA induces cell death in neutrophils, vital NETosis may occur through a more rapid signalling pathway (127). Fungal carbohydrates and proteins also induce NETosis in a short timeframe. The *C.albicans* derived cell wall component β -glucan were found to produce fast-response NETosis similar to those previously reported above (128).

In all cases, the authors highlighted this process being reactive oxygen species independent, stating this could be the explanation for the short window before NET release. The common ground between the works of Clark *et al.*, Pilsczek *et al.* and Byrd *et al.* is the receptor to which these stimuli bind. Toll like receptors 2 and 4 are shown to be involved in fungal host responses, not just bacterial, suggesting that this could be the shared signalling route for vital NETosis (129). It may well be that TLR2/4 signalling activates NET signalling

pathways much faster than other stimuli and would make sense given that the immune system is required to respond to pathogenic stimuli as fast as possible in order to eliminate threats.

1.3.3.5 Signalling mechanisms underpinning NET formation

As previously stated, NETosis has been observed through TLR4 signalling, PKC activation, resulting in NADPH oxidase activation. This section will summarise the various intracellular systems in place based on existing knowledge of these activation pathways, and how NETosis is activated through them.

PKC activation is shown to potently induce NETosis. Protein kinase C is a family of 12 kinases that are involved in cellular responses from a variety of agonists. Typically, PKC is activated by diacylglycerol production due to hydrolysis of the inositol phospholipids PIP₂ and phosphatidylcholine in response to extracellular signals (130-132). This hydrolysis also raises levels of Ca²⁺ to a sufficient level that allows these ions to bind to the inactive C2 domain of PKC, in addition to DAG binding the C1 domain of the enzyme (133). The binding of calcium to the C2 domain causes a conformational change in the C2 site opening up phospholipid binding site of the enzyme (133). The chelation of Ca²⁺ by the C2 domain changes the nature of the protein from repelling phospholipids, to becoming attracted to them, causing rapid translocation of PKC to the phospholipid membrane (134, 135). From here, PKC is cleaved and released into the cytosol in an active form (136). Nauseef *et al.* found that PMA treatment in neutrophils caused phosphorylation of the p47 and p67 subunits, resulting in association with *b cyt*, forming the NADPH oxidase complex (137). PKC-

mediated p47^{phox} translocation to the membrane has been further confirmed by Dang and colleagues, further explaining the mechanism through which PKC facilitates NADPH oxidase formation (138).

Superoxide produced via the respiratory burst is then able to generate high levels of anionic charge, which compensated by increase in intracellular K⁺ ions. These rapid shifts in ionic charge lead to release of NE and cathepsin G (139). In addition to degranulation, superoxide generation and neutrophil activation cause calcium release from the endoplasmic reticulum, as well as from extracellular sources, creating a high calcium ion environment in the neutrophil cytoplasm (140).

Work by Gray and colleagues found no sign of NETosis upon blocking of NADPH oxidase or protein kinase C with various inhibitors for either enzyme (141). The only agonist that neutrophils were treated with, however, was PMA. Phorbol esters degrade at a much slower rate than diacylglycerol, as well as forming irreversible membrane-bound forms of PKC that result in long term activation compared to DAG (142). This then poses the question... how biologically relevant is PKC activation in NETosis?

The PI3K/Akt pathway is crucial in induction of NETosis. DeSouza-Vieira and colleagues have demonstrated that inhibition of PI3K subunits PI3K γ and PI3K δ , as well as downstream activation of ERK from PI3K γ resulted in reduced levels of NETosis (143).

The pathway itself is activated through a vast array of stimuli, though in terms of relevance to NETosis, the toll-like receptors activate this pathway (144). Upon LPS binding to TLR2 and 4, receptor dimerization occurs, recruiting Toll-IL-1 resistance domain-containing adapter protein (TIRAP) by binding to PIP₂ (145).

TIRAP then anchors in the membrane, signalling MyD88 to be recruited from the cytosol to the TLR complex. Laird *et al.* found that MyD88 then facilitates the interaction of PI3K with the TLR complex (145). Class I PI3K kinases are kinases consisting of two subunits: p85, used to bind to proteins, and p110, used to phosphorylate phosphoinositol lipids (146). The p110 subunit on PI3K is then brought into close proximity of PIP2, causing phosphorylation into PIP3, phosphorylating phosphoinositide-dependent protein kinase 1, in turn phosphorylating Akt (145). The primary target of Akt is the mammalian target of rapamycin, or mTOR. McInturff *et al.* has shown mTOR as a mediator of NETosis through regulation of the hypoxia-inducible factor 1 α (HIF- 1 α) pathway (147). LPS induces activation of the HIF pathway, even in the absence of hypoxia, the condition through which this pathway is typically triggered (148). In the study of McInturff *et al.*, HIF-1 α was genetically knocked out in HL-60 leucocytes, and found to result in severely reduced levels of NET formation compared to wild-type cells (147). Additionally, this group was the first group to show regulation of HIF-1 α by mTOR in neutrophils. Unfortunately, a mechanistic means through which the HIF- 1 α regulates NETosis remains unclear.

Reports of Raf/MEK/ERK pathway activation have been reported, though this may serve to inhibit apoptosis in order for NETosis to proceed through NADPH oxidase activation (149, 150). Douda *et al.* have also shown a protective effect of Akt activation by diverting cells from apoptosis towards NETosis (151).

Autophagy has been reported several times in being required for NETosis (112, 121). The observations of vital NETosis by Fuchs *et al.* noted large vacuole formation after stimulation before membrane rupture and DNA release (121). These vacuoles were found to be autophagosomes at various stages of maturation (112). The size of the autophagosomes have been suggested to be

produced from the endoplasmic reticulum, with this loss of integrity further contributed to increased Ca^{2+} levels in the cytosol as a result of leakage in the endoplasmic reticulum (112).

1.3.3.6 Mechanisms of NET formation

As stated above, the key events regarding NETosis involve increase in Ca^{2+} levels and release of granule proteins. The point of convergence can be focused on activation of three key enzymes: myeloperoxidase, neutrophil elastase and peptidylarginine deiminase (PAD).

1.3.3.6.1 The role of myeloperoxidase in NETosis

Myeloperoxidase is stored in primary neutrophil granules. Upon neutrophil activation, MPO is released and enters the cytosol, primarily functioning to generate reactive oxygen species in reactions mentioned previously. Chemical inhibition of MPO through use of 4-aminobenzoic acid hydrazide has been reported to result in lack of NETosis (152). This has been further corroborated in patients completely lacking MPO, failing to make NETs in response to stimuli, though patients with MPO deficiency still produced NETs, but in a reduced capacity (117).

Papayannopoulos *et al.* has provided the greatest insight into the role of MPO in facilitating NETosis (153). Upon release from the granules into the cytoplasm, MPO translocates to the nucleus where it digests histones and binds to chromatin. Once bound, MPO facilitates substantial chromatin relaxation, resulting in decondensation of the fibres before forming NETs (153).

Interestingly, neutrophils stimulated with live pathogens did not require MPO in order to undergo NETosis, though this could be linked to the type of NETosis triggered (154). Bacterially induced NETosis appears to act independently of NADPH oxidase and given the intimate relationship between NADPH oxidase and MPO, it would make sense for this enzyme to partake in NADPH oxidase-independent NETosis.

MPO is also found extracellularly localised in NETs, and as such is used as a detection marker alongside DNA in immunofluorescence studies to confirm the presence of NETs.

1.3.3.6.2 The role of neutrophil elastase (NE) in NETosis

The role of NE in NETosis is not dissimilar to the role of MPO. Degranulation as a result of ROS production triggers the release of granular proteins (153).

Similarly to MPO, NE translocates to the nucleus from the cytoplasm during NETosis in a ROS-dependent pathway. Papayannopoulos *et al.* used the NE inhibitors GW311616A and serum leucocyte protease inhibitor to inactivate NE and observe and changes in levels of NET production (153). The study found that histone H4 was degraded by NE, not H3, which is typically found to be altered during NETosis (155). NE fully degraded H4, compared to other histones which were only partially degraded (153). The authors concluded that chromatin structure had an influence on the pattern of histone degradation, though whereas MPO facilitates chromatin decondensation, the role of NE appears to be involved in histone degradation (153).

1.3.3.6.3 The role of peptidylarginine deiminase in NETosis

Peptidylarginine deiminases are a family of enzymes of which there are 5 isotypes: PAD1, 2, 3, 4 and 6 (156). The function of these enzymes is to convert the amino acid arginine into a citrulline residue on proteins. This subtle change has a profound effect on the function of the protein. The deimination reaction neutralises the positive charge of arginine, into a neutral residue that serves to alter the tertiary structure of the protein (157). This change may affect protein-protein, protein-DNA and protein-RNA interactions. PAD4 has a nuclear transfer signal, allowing PAD to translocate from the cytosol to the nucleus and participate in gene regulation (158). Interestingly, PAD4 is integral to the process of NET induction, though appears to play no other role in general neutrophil function (118, 159). PAD4 is the most prevalent PAD isoform present in neutrophils and is involved in NET formation (160, 161). PAD4 is activated through exposure to high levels of cytosolic calcium, in the region of 100 times greater concentration than the normal resting Ca^{2+} cytosolic levels of $\sim 100 \text{ nM}$ (162). Interestingly, regulation of this enzyme was found by Neeli to be mediated by two isoforms of PKC, PKC ζ activating PAD, and PKC α inhibiting it (163). During neutrophil activation in response to stimuli *via* pathways previously described, the calcium level in the cytosol rises to substantial levels, triggering the adequate levels for PAD activation. Upon activation, PAD4 was found by Liu *et al.* to form a dimer, considered to be the active form of the enzyme. The monomer elicits $\sim 25\text{-}50\%$ of the activity of its' dimer counterpart (164).

Upon translocation to the nucleus, PAD citrullinates the arginine residues on histones, weakening the bonds between chromatin fibres and the histones they are bound to (165). Neeli found the primary target of PAD4 in the nucleus to be

histone H3, and that citrullinated histone H3 is also present in NETs that are released into the extracellular space (165). As histones are found to be potentially antimicrobial, Neeli proposes that the citrullinated variant may interfere with bacterial cell wall integrity (165, 166).

Between the activity of MPO, NE and PAD4, this triad of enzymes appears to be the key regulators of NET induction, though recent controversial opinions have been proposed that PAD4 does not in fact induce NETosis but occurs by a separate independent mechanism termed:- “Histone hypercitrullination” (111). Konig and Andrade argue that citrullination of histones and subsequent DNA release is due to membranolytic damage, not NETosis (111).

In conclusion, NETosis provides an additional weapon in the arsenal of neutrophils to kill pathogens. It is activated through a variety of means, and signals a plethora of intracellular pathways in order to achieve DNA release from the cell (167).

There has been great interest with how this process pertains to autoimmunity pathology. Given the dual-functionality of some proteins implicated in pathway activation, these may carry a detrimental element in certain autoimmune diseases. Given the involvement of PAD and MPO in NETosis induction, one of the most relevant autoimmune diseases that is proposed to result from aberrant levels of NETosis activation is rheumatoid arthritis.

1.4 Rheumatoid Arthritis

Rheumatoid arthritis belongs to a group of diseases that share a common aetiology in that immune system dysfunction drives the disease pathology. This dysfunction occurs from a breakdown in immune tolerance, the mechanism

through which the host immune system is programmed to not mount a response to host antigens. In RA, the joints of the victim are under continuous immune system attack, leading to degradation of the synovium due to prolonged inflammation in the joints.

Unfortunately, like other autoimmune diseases, RA lacks a definitive causative element that results in disease onset, merely risk factors and associations. Well established genetic factors are found to account for 50% of the risk factors for RA development (168).

1.4.1.1 Epidemiology and risk factors

Rheumatoid arthritis occurs at a frequency of 0.5-1% in the general population, with women being two to three times more likely to develop RA than men (169, 170). The incidence of RA is between 20 and 50 cases per 100,000 people in North America and Europe (171). Women on average develop RA around the age of 45, whereas men develop RA at a much older age, where the gap between the incidence of RA between men and women becomes more homogenous (F:M ratio of 4.84 at 40-49, to 1.65 at 70-79) (172).

As women develop RA at a younger age than men, sex hormones are thought to play a role in the earlier age of onset. The contraceptive pill appears to protect against RA, which may suggest that it is the cycling levels of sex hormones, not the high levels of certain hormones themselves, that may drive RA pathology (173, 174). Oestrogen enhances humoral responses on cells, further driving the suggestion that women also have a stronger immune system than men (175). The paradoxical question then arises of why the contraceptive pill appears to protect against RA? The only answer appears to be that as

progesterone is capable of eliciting an immunosuppressive effect, that this balance of progesterone and oestrogen must weigh in favour of progesterone, ultimately reducing the activity of immune system in women (176, 177).

Heritability is thought to be around 60%, and GWAS studies have shown over 100 genetic loci that are associated with RA (178-180). The largest genetic risk factor for RA is the shared epitope (SE) allele. The 'shared epitope' is actually a series of peptides encoded for as part of the HLA DRB1 gene, and is part of the antigen binding groove of MHCII, suggesting that mutations in the SE may lead to a malfunction in antigen presentation (181-184).

Other genetic risk factors in RA code for various enzymes and receptors in cells. A haplotype of the PADI4 gene, coding for the enzyme PAD4, has shown an increased risk for RA, as well as an association between the haplotype and antibodies to citrullinated proteins (185, 186). The protein tyrosine phosphatase non-receptor 22 gene (PTPN22) presents another important risk allele for RA, with polymorphisms of this gene resulting in an odds ratio of 1.6 for RA development (179). PTPN22 is a negative regulator of the Src and Syk family kinases, which are required to internalise and reprocess antigens bound to antibodies upon Fc receptor binding (187).

Two major environmental non-genetic risk factors for RA are smoking and inhalation of dust particles, through occupational exposure or general air pollution (188).

Smoking is a substantial risk factor for RA development (189). In a cohort of 700 cases and 850 controls, ever smoking was considered to provide an increased risk for RA with an odds ratio of 1.7 (190). Furthermore, a positive correlation was found between pack years of cigarette smoking and

seropositivity in diagnostic tests for RA, which focus on measuring levels of autoantibodies in the blood (191). This is even more pronounced in heavy smokers, who are 3.1 times more likely to test positive for RA than never smokers (191, 192).

1.4.1.2 Clinical presentation

Compared to osteoarthritis, RA occurs symmetrically in the joints of the human body. Swelling of the joints is common, which often results in inability to fully move the inflamed joints (193). Early stages of RA typically involve the joints of the hands and feet, often undetectable by radiological scanning (193). The key features of early stages are characterised by the “Three Ss”: which are early morning stiffness of longer than 30 minutes, joint swelling in more than 3 joints, and tenderness upon squeezing (194). If these clinical features are present, referral to a specialist should be advised. In later stages of RA, the larger joint spaces (knee, shoulder and elbow) become infiltrated with inflammatory cells resulting in further synovitis.

The consequences of RA are not restricted to joint inflammation. The constant activation of the immune system can affect the wellbeing of the patient through fatigue, fever and weight loss. Other organ systems may also be affected, with pulmonary involvement in RA usually manifesting as interstitial lung disease, with around 30% of RA patients reporting some form of lung disease as a co-morbidity of RA development (195).

Another hallmark of RA is development of rheumatoid nodules. These subcutaneous nodules are the most common feature of RA, but are not involved in the joint pathology, typically appearing on the hand and arms (196)

and also in the lungs (197). Nodule formation appears to affect roughly 10% of RA patients, and although inconvenient, are not normally of concern for patient health. Paradoxically, methotrexate and possibly tumour necrosis factor inhibitors can increase nodule development and subcutaneous nodule formation has been linked to increased cardiovascular events in RA patients (198). Surprisingly, very little is known about the composition of these nodules or if they are a source of pro-inflammatory mediators.

1.4.1.3 Testing for RA

The criteria that is used to diagnose RA has been developed between the American College of Rheumatology and the European League Against Rheumatism to create Rheumatology 2010 rheumatoid arthritis Classification Criteria (199). This new framework serves to aid in early diagnosis, as prior criteria used to define RA by late-stage symptoms of the disease (199). This criteria assigns a score based on serology and physical examination results, to ultimately diagnose a patient with RA.

C-reactive protein (CRP) is used as a measure of active inflammatory activity in RA. It is an acute-phase protein that is produced as a by-product of inflammation (200). CRP is thought to work similarly to antibodies, in that it is capable of binding to Fc receptors on innate cells, activating complement, and initiate proinflammatory cytokine release (201). The level of CRP is used to calculate the disease activity score, (DAS)28. DAS28 can also be calculated through measurement of erythrocyte sedimentation rate, though DAS28-CRP is thought to be the better tool as CRP is more sensitive to rapid changes in disease activity (202). Ultimately, both formulae use the serology from the

respective marker alongside feeling 28 joints of the body, to check if they are tender or swollen (202).

The core hallmark of autoimmune diseases is the presence of autoantibodies. Each autoimmune disease typically has a characteristic autoantibody indicative of the disease. Importantly, the reasons why these autoantibodies are generated by plasma cells remains unknown, driving the need to understand why autoantigens are generated, in order to develop more efficient methods in early treatment of autoimmune disease.

Anti-Citrullinated Protein Antibodies (ACPAs) are autoantibodies specific to RA. ACPAs are used to diagnose RA prognostically with varying levels of assay sensitivity and specificity through measurement of antibodies to cyclical citrullinated peptide (anti-CCP). Aggarwal *et al.* reviewed various commercially available anti-CCP assays, the sensitivity of anti-CCP assays ranged from 56-78%, and specificity between 87-99% (203). These antibodies can be detected in RA patients long before symptoms begin to appear, though these observations were discovered before the criteria was updated in 2010, therefore the observation of ACPAs up to 14 years before RA symptoms develop (204). The highly specific nature of ACPAs is important, as ruling out patients suspected of RA is integral, to prevent highly immunosuppressive and expensive therapies from being used on those that do not need it.

The other diagnostic biomarker in RA is rheumatoid factor (RF). RF is an autoantibody immune complex, comprised of two immunoglobulins IgG and one other that can exist in three forms: IgM-RF, IgA-RF and IgG-RF, and exist in that level of prevalence, with the IgM-RF typically being the dominant complex in serum (205-207). RF is not restricted to RA pathology; it can also be used in

the diagnosis of systemic lupus erythematosus and primary Sjögren's syndrome (208, 209). It exists merely as tool to complement the other assays used in diagnosis of autoimmune disease, and as such has a sensitivity and specificity of 75% and 85%, respectively (210).

More recently, anti-carbamylated protein antibodies (Anti-CarPs) have been identified in RA and show promise of a new biomarker in RA diagnosis (211, 212). The sensitivity and specificity of Anti-CarPs is lower than ACPAs at 26-44% and 89-95%, respectively (213, 214). Although the lower sensitivity of the assay does not make it suitable as a primary diagnostic assay. It may prove useful in RA patients who appear to be RF-negative and ACPA-negative due to its high specificity, and may prove useful as an additional tool in overall RA diagnosis and management.

Autoantibodies to other proteins which may be citrullinated in RA have been detected in seronegative RA patients, such as citrullinated calreticulin (citCRT). Citrullinated calreticulin binds to the SE component of the MHCII as discussed in **Section 1.4.1.1**, resulting in immune system dysfunction (215). Anti-citCRT antibodies have been previously detected in 49% of patients with RA compared to 9% of patients who displayed antibodies to the unmodified form (216). Furthermore, it was demonstrated that anti-citCRT antibodies can capture 35% of a cohort of RF –ve ACPA –ve RA individuals, providing further promise in detection of this disease (216).

1.4.1.4 Pathophysiology

RA develops in part as a result of multiple cell dysfunctions, with the central focus of this being a result of aberrant post-translational modifications: citrullination and carbamylation of cell proteins. A summary of how this process occurs can be shown in **Figure 1.7**.

Anti-citrullinated protein antibodies (ACPA's) have long been recognised as a key feature of RA. As mentioned earlier, citrullination is mediated by the PAD family of enzymes. The production of citrullinated proteins in RA pathology is well documented, but PAD enzymes also play a role in NETosis (217-222). Citrullination is an irreversible process through which the amino acid arginine is replaced by citrulline on proteins. This change in residue negates the overall charge of the protein, leading to implications for overall protein structure, or possibly function (223). The large portion of ACPAs in RA are directed against abundant structural proteins such as fibrinogen and vimentin (224, 225).

Although ACPAs are seen as a biomarker of RA, their pathophysiological role is unclear. Although initially reported by Krishnamurthy *et al.* to initiate the inflammatory response in RA through osteoclast-mediated IL-8 release, the authors corrected their article years later to clarify that this was not the case, casting doubt on the role of ACPAs as a potential driving force in joint inflammation (226, 227). Trouw *et al.* demonstrated the ability of ACPAs to activate complement system years before (228). The role of complement activation to antibodies typically results in immune complex clearance, though antibodies bound to tissue-bound antigens typically trigger the membrane attack complex and subsequent cell death, along with immune cell chemotaxis (229,

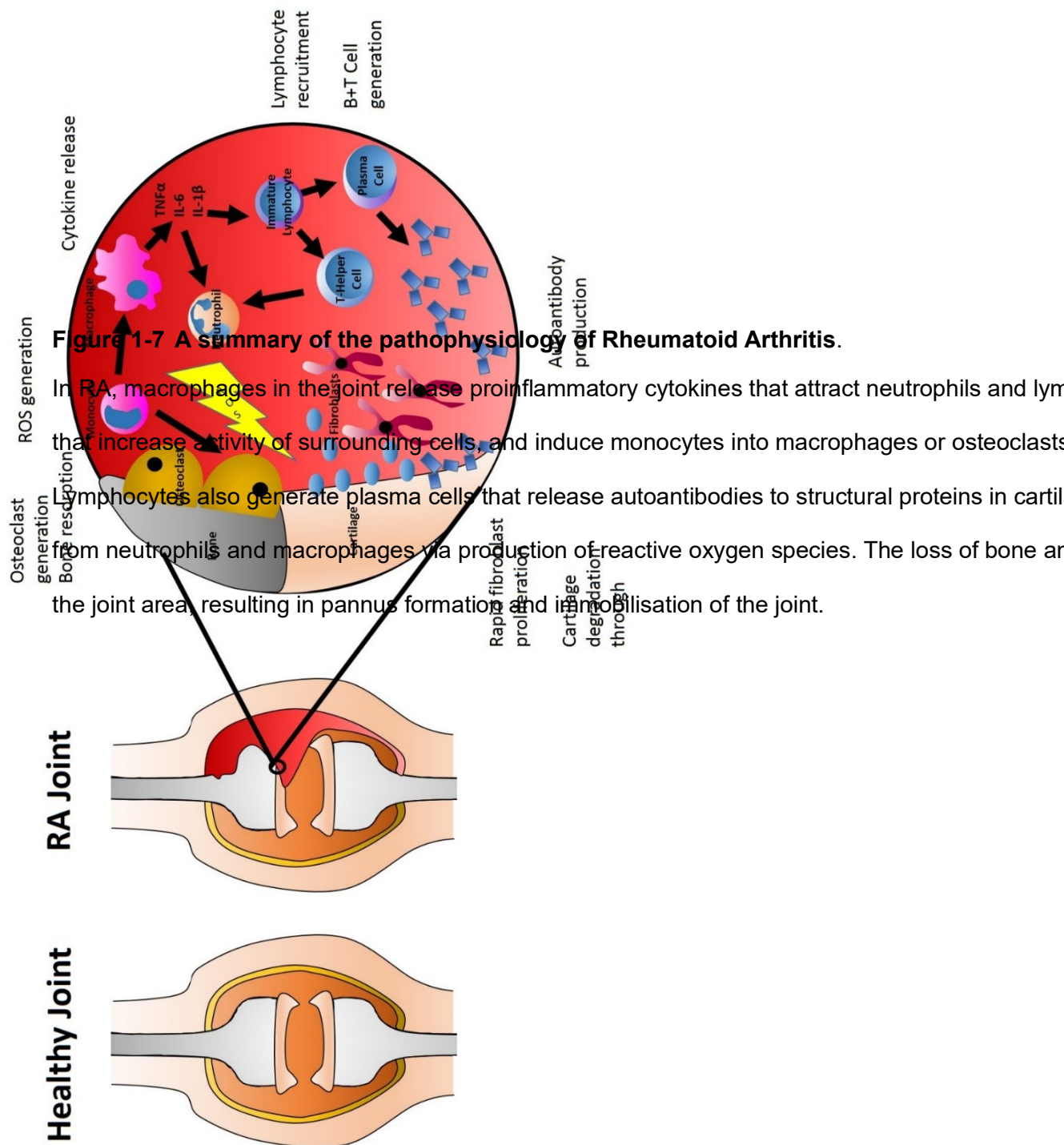
230). Taken altogether, this may suggest that ACPAs binding the aforementioned citrullinated structural proteins above in the joint could help facilitate and potentiate the inflammatory process through complement activity.

Similarly to citrullination, carbamylation has recently been found to play a similar role in RA pathology, with anti-CarPs being detectable in similar levels to ACPAs in early RA patients (211, 212, 214). Unlike ACPAs however, carbamylation is a chemical reaction principally between cyanate and lysine residues on proteins (231). Although the reaction is chemical, myeloperoxidase catalyses the reaction between thiocyanate (inhaled in cigarette smoke) and H_2O_2 to produce cyanate.

When this level is raised during bouts of inflammation, the free cyanate is free to react with lysine to form carbamyl-lysine (232). This alters the function and structure of the protein, similarly to citrullination.

Through citrullination and carbamylation, the host protein may be marked as a neo-antigen, in that the process creates a variant of the protein that the immune system has not raised tolerance against (221). During cell death the newly modified proteins produced in dying cells are released into the extracellular space. Here, they are recognised by innate cells as an antigenic molecule, prompting internalisation and processing and subsequent expression through MHCII. From here, dendritic cells present the antigen to the adaptive immune system, resulting in autoantibody generation.

Carbamylation of vimentin induces autoantibody production by B-cells, as a result of cigarette smoke, though no association between smoking and positivity of anti-CarPs has been established to date (233).



In RF, Igs bind the Fc region of IgG in the CH2 domain, through mechanisms currently unknown (234). What is known in RA pathology however, is that these three autoantibody classes circulate in the body long before RA pathology begins, increasing slowly before joint damage occurs (235). The joint is relatively uninvolved in early stages of RA, suggesting that autoantibody development occurs at a site away from the synovium (236).

The link between autoantibody development and the initial stages of synovial inflammation remains elusive (237). Multiple theories have been suggested, such as deposition of immune complexes in the physical spaces of the body, or epitope switching of autoantibodies resulting in the non-citrullinated targets in ACPAs being targeted (235). However, after the initial inflammation takes place, the end result is that the joints become a highly active inflammatory environment.

The synovium consists of synovial fibroblasts, alongside synovial macrophages. In RA, rapid proliferation of fibroblasts occurs, to a density of 5x the normal thickness (238). These fibroblasts remain persistently activated, and release factors that degrade the surrounding cartilage in the joint (239). In the initial stages of RA, resident macrophages release the proinflammatory cytokines $\text{TNF}\alpha$, IL-1 and IL-6 and attract other immune cells towards the joint (240). This attracts neutrophil and lymphocytes towards the synovium in later stages of the disease (241). CD4^+ cells are the most prevalent lymphocyte subtype in the RA joint, which further augment macrophage function and promote ROS production and joint degradation (242). CD4^+ cells also stimulate B-cell proliferation and further drive plasma cell differentiation, which in turn produce more autoantibodies that cause further inflammation (242). The final function of CD4^+ cells in the joint is to promote generation of osteoclasts that degrade bone

tissue, and contribute to pannus formation (243, 244). The pannus is the term given to the overgrown fibrous tissue that replaces cartilage and bone as a result of the synovial fibroblast activity.

1.4.1.5 The role of neutrophils in RA pathology

Neutrophils are thought to play an essential role throughout all stages of RA. Immune complexes in the joint activate neutrophils and promote degranulation resulting in ROS production and release of enzymes that degrade the structural proteins in the synovium (245). Synovial fluid of RA patients routinely contains high levels of neutrophils compared to other cell types, which in turn exacerbate the inflammatory environment of the joints causing further damage (246).

Posttranslational modifications of proteins found in RA by the process of citrullination and carbamylation, are mediated by PAD and MPO, respectively. Neutrophils contain both of these enzymes, and of greater interest is the dual role of these enzymes in NETosis. NETosis has been suggested as a potential mechanism through which posttranslational modification of host proteins occurs (219, 247, 248). Neutrophils isolated from RA patients undergo spontaneous NETosis, in addition to more enhanced NETosis when stimulated with PMA (249). Chowdhury also found higher mRNA expression of MPO and NE, as well as higher levels of citrullinated histone H3 (249). PAD4-DNA complexes were also detected in the supernatants of neutrophils stimulated with PMA, demonstrating the ability of PAD to be released extracellularly in NETs (249). This novel finding along with the discovery of MPO in NETs, suggests that this process could be a crucial pathway through which RA associated autoantigens may develop (152). IgA-RF further induces NETosis in RA neutrophils,

suggesting that as well as the initial stages, NETosis may be generating citrullinated and carbamylated proteins in a continuous feedback loop (205, 206). In the context of RA, Corsiero *et al.* isolated B-cells from RA patient synovial tissue and tested them for reactivity to citrullinated proteins (250). They found 40% of B-cells in the joint produced antibodies reactive against citrullinated proteins, with the largest portion of these antibodies having reactivity to citrullinated histones H2A and H2b, demonstrating NETosis as a mechanism for RA pathology.

1.4.2 The relationship between lung disease and autoimmunity

The lungs have been proposed as the initial site of autoimmune disease development (251). The constant exposure of the lungs to pathogens in the environment highlights the need for a strong immune presence to defend against infection and disease. In the lungs, several specific defences exist.

Alveolar macrophages reside in the lungs to deal with day-to-day pathogens that may persist in the lungs. Their function is similar to conventional macrophages, with phagocytosis, ROS production, and proinflammatory signals to attract other leucocytes during high levels of pathogen loads (252). The interplay between antigen presenting alveolar macrophages and B cells is crucial in the maintenance of pulmonary immunity. Bronchial associated lymphoid tissue (BALT) provides a reservoir of B-cells close by in germinal centres that produce vast quantities of IgA (253). IgA a broad spectrum binding class of immunoglobulin, and is secreted in mucosal tissues of the body in higher daily amounts compared to IgG (254). In mucosal sites, dimeric IgA is the most common form of synthesised IgA, which is transported through

epithelial cells into the bronchial lumen to bind pathogens that are subsequently trapped by mucus.

On epithelial surfaces, release of mucins from goblet cells is integral to pulmonary immunity. Mucins are highly adhesive glycoprotein molecules that bind to and trap inhaled pathogens and toxins, and are continuously wafted up out of the lungs and into the gullet by ciliary fibres (255). Disruptions in mucus clearance can lead to opportunistic infections, as bacteria trapped in the mucus are free to infiltrate the lungs and then infect the body. Several lung diseases highlight mucociliary clearance as a causative element to disease pathophysiology, such as cystic fibrosis and bronchiectasis.

Cystic fibrosis (CF) occurs from defects in the CTFR gene, which regulates the transfer of sodium and chloride ions, as well as formation of molecular complexes on the plasma membrane (256). The impaired ion exchange system leads to less liquid being present in the lumen of the lungs, producing thicker mucus. Thicker mucus is harder to be cleared by cilia, leading to recurrent, serious infections in the lungs (255).

Bronchiectasis (BR) is a chronic lung disease characterised by the permanent dilation of the bronchial airways (257). The dilation may be mediated by loss of elastin in the lumen, with the initial damage occurring during adolescence as a result of a serious bacterial infection (258). Bronchiectasis can be classified as CF or non-CF bronchiectasis, as CF sufferers frequently suffer from BR pathology, prompting clinicians to first rule out a diagnosis of CF before diagnosing a person with non-CF BR. Patients suffer from the same problems as CF patients, with inability to clear mucus resulting in frequent infections (258). These frequent infections result in a cycle of infection, inflammation, lung

damage, poor clearance of mucus, and further infections, all amongst the backdrop of increasingly poor lung function. Mortality from BR occurs in roughly 30% of BR patients, occurring in the later, more severe stages of the disease (259).

Neutrophil infiltration is a key driver of BR pathology (259-262). In order to clear trapped pathogens in the lungs, neutrophils enter the lung as a result of IL-8 release from alveolar macrophages alongside TNF α release from epithelial cells (262). Inhibitors of the IL-8 receptor have been reported to reduce the neutrophil presence in sputum counts, though interestingly did not improve any clinical outcomes in BR patients (263).

Once in the lungs, the highly potent antimicrobial activity of neutrophils is commenced. Primary immune function is elicited in the form of phagocytosis of pathogens, opsonised by secretory IgA produced by plasma cells in the local area from BALT. In BR, neutrophils have been shown to be reprogrammed and last longer before undergoing apoptosis, release higher levels of antimicrobial proteins and are incapable of efficient phagocytosis of pathogens (260, 264).

The ability of *Pseudomonas aeruginosa* to form biofilms in the lung, when faced against faulty neutrophil phagocytic mechanisms is one of the reasons that chronic infections in BR most commonly occur from *P. aeruginosa* (265).

Production of ROS in addition to degranulation creates a highly damaging environment towards lung tissue however, with neutrophil elastase degrading the elastin fibres in the lung, driving the aforementioned cycle of lung function degradation (264).

Similarly to neutrophils in RA, BR neutrophils have also been shown to have enhanced capacity to produce NETs (266). These NETs have in turn been

shown to induce proinflammatory cytokine release, as well as cell death in some cases (267, 268).

Higher levels of NETs, proinflammatory cytokine release from both bacteria and NETs, and loss of integrity of lung structural proteins essentially turns the lungs into a never-ending source of inflammation, and the constant activity of the immune system provides a perfect storm that has been hypothesised to be the initiating site for autoimmune disease development.

Bronchiectasis has been associated with rheumatoid arthritis since 1967 (269). The incidence of both BR and RA (BRRA) is thought to occur in roughly 30% of RA patients, suggesting BR as either a cause or a consequence of RA pathology (270). Cigarette smoke has been proven to be one of the biggest risk factors for RA onset, with PAD4 levels being increased in the bronchial lavage fluid and lung tissue of smokers, leaning into the idea that BR leads to RA, as opposed to vice versa (189-191, 271).

RA patients with lung disease have been shown to have higher ACPA titres compared to those without (272). This may be attributed to higher levels of citrullinated protein in patients with lung disease. RA patients show the presence of inducible BALT, which are additional lymphoid follicles that develop as a result of infection, compared to BALT which exists as a protective lymphoid follicle (273). In this study, RA patients had ACPA producing B cells in the iBALT in the lungs, suggesting that ACPA production can occur in both the lungs and the joint (273).

In 60 newly diagnosed RA (<12 months), Wilsher found correlations between ACPA titres and lung function, though no correlation with disease activity.

Bronchiectasis was found to be present in 48% of the cohort, with 55% vs 41%

having ever/never smoked (274). The high prevalence of BR in this cohort, which is otherwise a rare disease affecting 21-35 people per 100,000 population, strengthens the argument that BR is a risk factor for RA (275).

Reynisdottir *et al.* corroborated these lung abnormalities in newly diagnosed RA patients, being present 63% of ACPA positive RA patients, compared to 37% of ACPA negative RA patients, and 30% of healthy controls (276). Lung biopsies of this cohort stained positively for citrullinated proteins in the ACPA positive patients, as well as finding ACPAs in lavage fluid in the same patients (276).

Perry *et al.* propose the “two-hit hypothesis” for RA pathogenesis (**Figure 1.8**) (277). Based on existing evidence, the lungs are the initiating site of RA through immune system activation in the lungs.

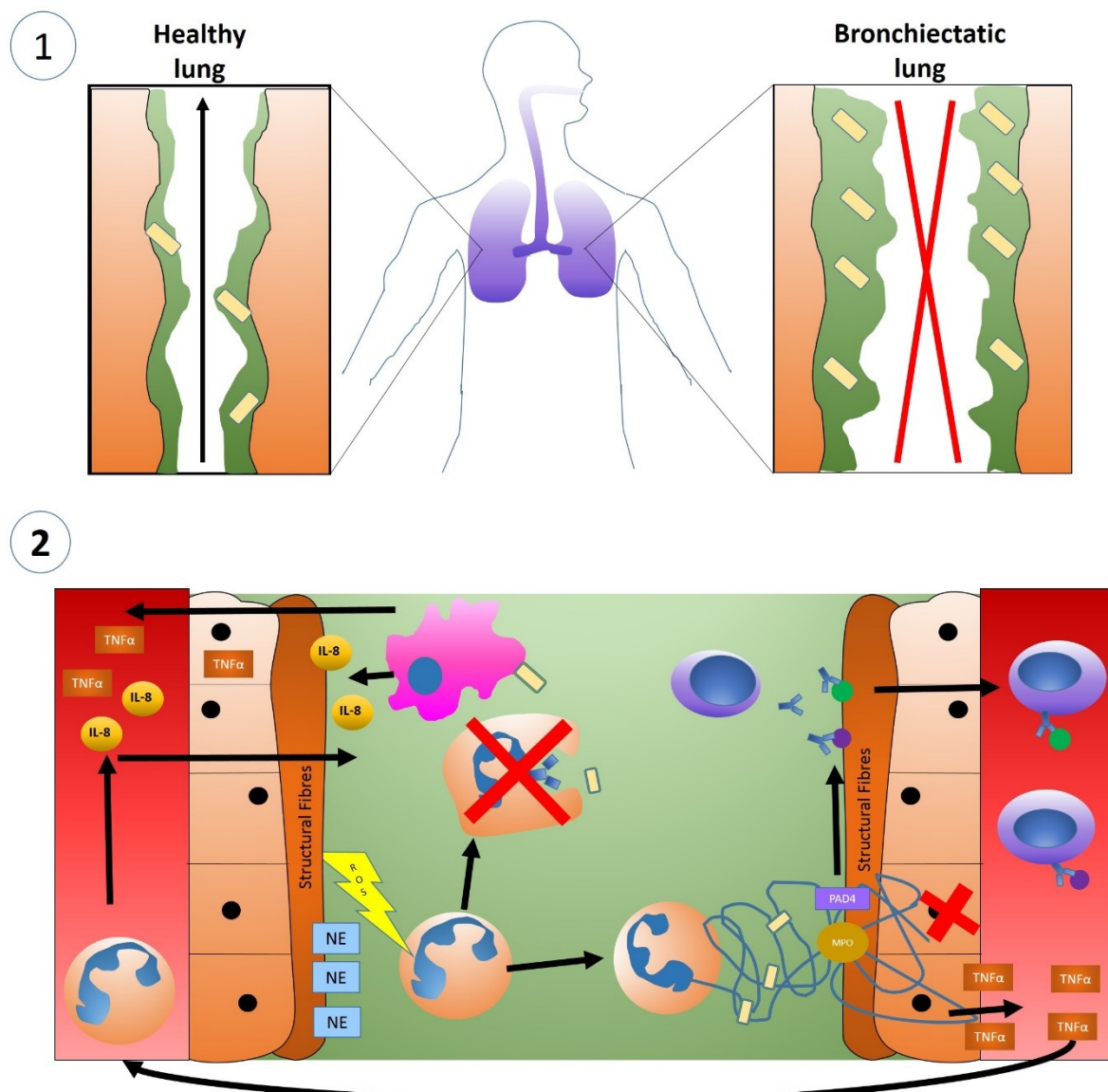


Figure 1-8 A hypothesis for RA autoantigen formation in bronchiectasis.

1) In bronchiectasis patients, the airways are dilated, leading to ineffective clearance of pathogens trapped in mucus. 2) Trapped pathogens cause cytokine release from alveolar macrophages and epithelial cells. These cytokines attract neutrophils that migrate into the bronchial lumen. Neutrophils are incapable of engulfing pathogens, and so undergo NETosis to clear the pathogen in addition to release of reactive oxygen species and neutrophil elastase that damages lung tissue. The release of myeloperoxidase and peptidylarginine deiminase in NETs into the extracellular space causes posttranslational modification of structural proteins in the lungs that results in autoantibody formation by B cells found in bronchial lymphoid tissue. These circulating cells then travel around the body and upon encountering similar proteins in the joint, commence an autoimmune attack.

This activation arises from both infectious and environmental stimuli, resulting in citrullination/carbamylation of proteins through neutrophil enzymes (PAD and MPO). Along with NETosis, extracellular presentation of these antigens gives rise to autoantibodies that result in memory B-cell formation, as a “first-hit”. In the “second-hit”, inflammation in the joint that occurs from trauma, where once again, modified proteins are produced as a by-product of immune system activity. The B-memory cells that contained the autoantibodies then recognise the modified structural proteins and expand, resulting in an immune response to the joint.

Bronchiectasis fits nicely into this hypothesis, with BR being a disease of recurrent infections, therefore frequent bouts of inflammation. NETs are involved in RA development, and neutrophil dysfunction has been reported in both BR and RA (260, 266, 278-280). Although citrullination has long been the focus of RA pathogenesis, carbamylation of extracellular proteins by neutrophils occurs irrespective of NETosis, further corroborating the notion that neutrophils are the driving autoantigen formation in RA (281).

Further examination into bronchiectasis and rheumatoid arthritis by Perry used conventional testing for RA on BR patients, measuring RF and ACPA titres (282). Of 122 BR patients tested, 25% of the cohort were positive for RF, with 61% of the cohort having never smoked. 4 patients who tested positive for RF also displayed very strong positivity for ACPAs (282). Of these 4 patients, 2 proceeded to develop RA, and Perry *et al.* were the first group to demonstrate ACPA positivity in BR. This cohort was examined further in a follow up study, comparing the original BR cohort against BERRA patients, and RA patients with no history of lung disease (283).

The BRRA cohort of this study were found to have significantly higher DAS28, RF and ACPA titres compared to the RA alone cohort. This BRRA cohort also had low levels of smoking recorded, suggesting BR as the driving factor for autoantibody positivity (283).

Sadly, no study exists that examines neutrophil function in BRRA patients, which would prove invaluable to determining any overlap between these two diseases. It is apparent that NETosis has been the focal point of research for good reason, with most well-studied diseases coming under scrutiny once again due to the revelation of this process in 2004. Key pieces of the puzzle for breakdown of immune tolerance in RA are slowly being unravelled. The evidence of NETosis induction through cigarette smoke exposure could open the door to finding out the causative stimulus for the greatest environmental risk factor in RA (123). Cigarettes are composed of a multitude of toxic compounds, and so it is of great importance to elucidate the NET-inducing element in cigarettes, in the hope any other routes of exposures in the population can be examined that may lead to RA development.

As previously mentioned, cadmium is present as a contaminant in cigarette smoke, and inhalation of cadmium is a frequent occupational exposure for industrial manufacturing, with recent research suggesting cadmium as a causative agent for RA.

1.4.3 Cadmium exposure as a risk for RA pathogenesis

Silica exposure, or dust inhalation has recently emerged as a risk factor for RA (284). As men appear to have a later age of onset in RA than women, it suggests that cumulative environmental stimuli may be the cause of RA

development. Stolt and colleagues found in a case-control study that silica exposure had an odds ratio of 2.2-3.0, depending on age, and exposure to silica (284). This has further been corroborated by Blanc *et al*, who found that foundry workers exposed to silica who had been occupationally exposed had a 57% increased risk for RA (285).

More recently, inhalation of nanoparticles, not just silica, has been suggested as a contributing factor to RA pathogenesis. Textile dust has also shown a strong correlation with RA patient serology, with those having ever been exposed to textile dust having an odds ratio of 2.8 for RA pathogenesis (286). Research by Murphy *et al*. found that in a cohort of 726 male RA patients in the south west of the UK, 75% had occupational exposure to dust for over a year, in addition to 77% of the cohort having ever smoked (287). Interestingly, RF levels were statistically significantly higher in both smokers and non-smokers with a history of dust exposure compared to those never exposed (287). The authors propose that nanoparticle inhalation provides a surface for cigarette smoke to adhere to, to further induce and prolong inflammation induced from smoking (288, 289). Nanoparticles and fibres are able to penetrate deep into the lungs and cause inflammation (290). This combination of nanoparticle inhalation and cigarette smoke may provide a system whereby the lungs are subject to constant sources of inflammation, which could provide a system of dysfunction through persistent over-activation, resulting in aberrant levels of NETosis, resulting in protein modifications in a similar mechanism to those proposed in bronchiectasis.

In the context of RA, cadmium may pose an additional mechanism for disease pathology through increasing the rate of bone destruction. Chen treated rats with various doses of cadmium for 12 weeks and performed histological

analysis on bone tissue after killing them (291). Cadmium exposure was found to reduce the bone mineral density in rats, as well as increasing levels of osteoclasts around the bone (291). In RA, monocyte/macrophage involvement is rather substantial in terms of destructive outcomes, but these cells also have the capability to transform into osteoclasts (292, 293). Taken altogether, this may provide a possible route for cadmium to contribute to RA pathology.

1.5 Effect of cadmium on immune system function

Research on cadmium exposure in the context of immunity has focused primarily of mouse models rather than *in vitro* work with human cells.

1.5.1 Lymphocytes

Borella *et al.* found that healthy B-cells exposed to cadmium at very low concentrations (50nM-1µM) resulted in increased immunoglobulin production in resting cells, but not B-cells that were activated (294). Interestingly, at concentrations of 10µM or higher, immunoglobulin production was markedly decreased in both stimulated and unstimulated B-cells, posing a risk for high-exposed individuals. If high doses of cadmium inhibits antibody production in B-cells, this may open up individuals to a diminished immune response in response to infections (294). The authors also found high levels of cadmium in the nucleus of cadmium-exposed lymphocytes, compared to the cytoplasm.

Daum *et al.* investigated this further, and found that CdCl₂ exposure on healthy B-cells inhibited RNA and DNA synthesis, with cadmium exhibiting an IC₅₀ for this at around 30µM, as well as arresting the cell cycle within 2h of exposure (295). In live rats, Kataranovski *et al.* found similar findings, with the proliferative capacity of splenic lymphocytes being reduced after being dosed with various

concentrations of cadmium (296). Notably, LPS induced expression of MHCII was inhibited by CdCl₂, further adding to the idea that cadmium suppresses host immunity, with the authors suggesting that the PKC pathway may be inhibited by cadmium (295). In terms of immunoglobulin production, IgG production was substantially affected by cadmium, with LPS-treated lymphocytes producing significantly less immunoglobulins at as little as 18µM of CdCl₂.

A study on the effect of cadmium exposure on rat lymphocytes was published in 2004, contradicting previous results, showing that as well as reductions in B and T-cell populations at low doses, higher doses appeared to increase general lymphocyte levels (297).

A more long-term examination of cadmium exposure on rats by Yuan *et al.* looked at a daily 0.5mg/kg treatment for 12 weeks in the context of lymphocyte viability, to attempt to explain the results found by Lafuente *et al.* (297, 298). Daily exposure of 0.1µM CdCl₂ for 12 weeks resulted in increased rates of proliferation, though doses of 0.5 µM and 1µM significantly reduced cell growth over a 48h period. Additionally, white blood cell volume was found to be significantly increased, though no effect on granulocyte or monocyte was observed, suggesting that this increase is most likely lymphocyte driven (298). In a model of viral infection in mice, lymphocyte levels were found to be significantly elevated in mice exposed to cadmium and the H1N1 virus, compared to either cadmium or the virus alone (299).

1.5.2 Monocytes

Macrophages appear to tolerate cadmium at rather substantial levels, though lose function of certain processes at much lower doses. Coin and Stevens

treated alveolar macrophages isolated from rabbits with cadmium and calculated an LC50 of 200µM in these cells (300). Phagocytosis of yeast cells was found to be inhibited at around 31µM, around the same concentration when the alveolar macrophages started to show morphological changes in cell shape (300). This study has several issues, however. The lethal concentration that was calculated to be an LC50 of 200µM was extrapolated from just 4 different treatment concentrations, which is an insufficient number to adequately calculate an exact dose at which cytotoxicity occurs. Additionally, this study predates the invention of flow cytometry, and so the means of assessing phagocytosis, by counting 50 macrophages on a slide and examining labelled yeast internalisation, of which the EC50 for phagocytosis inhibition was calculated, was from a treatment conferring a standard error between 40-90% of the control sample. One may argue that there are differences between rabbit and human alveolar macrophages in terms of phagocytic capability, though Nguyen concluded that there are relatively similar mechanisms and stimuli that are shared between these two species (301).

In the same viral infection study stated above, monocyte levels were found to be significantly raised in mice exposed to 1mg/L CdCl₂ then infected with H1N1, compared to cadmium or H1N1 alone (299).

Cox *et al.* explored the effect of macrophage function when exposed to cadmium before stimulation with LPS. In this study, THP-1 monocytes were differentiated into macrophages from PMA stimulation. In addition to this, primary rat alveolar macrophages were isolated and both were incubated with various concentrations of cadmium, before being challenged by 100ng/ml of LPS, then examined the effects on the cell biology (302). Cadmium preincubation induced gene expression of IL-6, IL-8 and TNF-α in monocytes

after LPS treatment more so than no cadmium treatment, though the opposite effect is seen in primary macrophages (302). Cytokine expression in both untreated, and between 0.1 to 1 μ M cadmium treated macrophages retained similar levels of gene expression that significantly dropped in a dose-response manner after 2.5 μ M cadmium pretreatment (302). Macrophages were also shown to release significantly lower levels of these cytokines when challenged with LPS after a 12h incubation with 10 μ M cadmium, though in the case of TNF- α , a significant reduction was also observed after a 4h treatment. Cox *et al.* also found that cadmium treatment in mice lead to increased activation of the NF κ B pathway in monocytes yet inhibited in macrophages upon stimulation with LPS.

Jin and colleagues confirmed these findings in a different cell line, RAW264.7 cells, though used a smaller window of cadmium concentration (0.1-3 μ M compared to 0.1-100 μ M in the other study) for a 24h period, compared to overnight in the study by Cox *et al.* (303). The macrophages were stimulated with a stronger dose of LPS (1 μ g/ml vs 100ng/ml) for 6h and 24h, versus 4h and 12h. Cell viability was shown to be significantly affected at 3 μ M. Jin's findings were in line with Cox *et al.*, who also found a dose-response mediated decrease in TNF α , IL1 β and IL6 release (303).

Oxidative stress is induced by cadmium, resulting in cell death (304-306). In macrophages, cadmium induced apoptosis from concentrations as low as 5nM over a 48h incubation period (307). This study found significant increases in incidence of early and late apoptosis, however this shift was from 0.8% to 2% in terms of annexin V positivity, and 2.5% to 3.5% annexin V/PI positivity. While statistically significant, the question of biological relevance of this finding comes in to question. The more interesting finding from this study was that a significant dose-response increase in mitochondrial ROS generation was observed in

macrophages treated with 5nM-2µM of cadmium whilst reducing mitochondrial membrane potential in a similar fashion (307).

1.5.3 Neutrophils

Similarly to most other immune cell studies, very little data explores the effect of cadmium on neutrophil biology. Kataranovski *et al.* found that granulocytes in live rats exhibited signs of activation, as well as increased survival signals (296). This was followed up by the same group, who treated rats with 0.5 or 1mg cadmium /kg body weight, and examined lung homogenates from sacrificed rats (308). Upon cadmium treatment, cell numbers were calculated from enzyme digestion to determine cell infiltration in the lungs. Neutrophils numbers were significantly increased in both cadmium-treated mice after 24 hour exposure, though neutrophil infiltration is still possible up to 48 hours after cadmium treatment (309, 310). MPO levels in the lungs also increased, though this is not surprising, given the elevated levels of proinflammatory cytokines that are produced by monocyte and lung epithelial cells that are strong chemoattractants for neutrophils. Elevations in both neutrophil cell numbers in the lungs, along with increases in MPO level signify that neutrophil involvement is present in the lungs in response to cadmium inhalation. Although macrophages are also sources of myeloperoxidase, neutrophil granules account for the majority of MPO in the body (309).

CD11b expression is increased upon cadmium treatment, potentially as a mechanism to bind to ICAM-1, a binding ligand of CD11b that is upregulated on hepatocytes after cadmium treatment (310).

Cadmium does not induce the respiratory burst in rat neutrophils treated with 1mg/kg for 48h, although in vitro experiments have shown evidence of ROS production from 2.5µM of CdCl₂ (311, 312). The authors of this study calculated the proportion of cells positive for DHR123, stating that cadmium diminished the amount of positive cells when treated with 100ng/ml PMA (311). This impediment to ROS production may be due to cadmium-induced p47phox phosphorylation, which could attenuate the conventional respiratory burst when stimulated with conventional molecules such as fMLP (313).

Chemotaxis is also affected by cadmium, with neutrophils of cadmium-treated mice exhibiting lower levels of ERK 1/2 phosphorylation, as well as reduced migration capacity when stimulated with fMLP (312).

Phagocytic ability of neutrophils has not been examined since 1978, when it was shown that cadmium inhibited the phagocytic capacity of mice neutrophils and alveolar macrophages, although it did not affect the ability of these cells to kill the internalised particles (314). This technique used light microscopy and counting of ingested particles, which is prone to inaccuracy.

1.5.4 Cadmium as a contributor to autoimmunity

Based on the literature shown above, it is evident that cadmium is both proinflammatory and immunosuppressive. Rat and mouse models of cadmium exposure note increases in white blood cell volume, though most notably macrophage and neutrophil populations. This suggests a heavy innate cell involvement in responding to cadmium entering the lungs. The cadmium present in the pulmonary environment diminishes the general functions of these cells, as research demonstrates that upon stimuli with molecules such as

endotoxin or fMLP, functional capacity is reduced. This then leads to the question: how could cadmium exposure possibly lead to RA development?

Cadmium may not directly induce the breakdown of immune tolerance. What remains important to consider however, is that the high cadmium level in RA patients shows that it contributes to pathology somehow (315). Silica and dust exposure is a risk factor for RA, and this was elaborated by suggesting that these molecules provide a deeply penetrating absorber of cadmium ions (316, 317). Smoking provides a continuous low-dose into the lungs, which can only be worsened through nanoparticle inhalation.

Here, cadmium induces chemoattraction of various immune cells to the lungs through induction of proinflammatory cytokines. Once in the lungs, immune system function is dampened down to cause dysfunction. This dysfunction may lead to ineffective clearance of pathogens in the lungs, predisposing an individual to infections, of which smoking is known to cause (318). Neutrophils are thought to undergo the process of NETosis when conventional means of antimicrobial activity are insufficient, typically due to pathogen size (319). It is unknown if NETosis is a last-resort for the cell when all other mechanisms are inadequate. NETosis can be induced from a variety of pathogenic stimuli and has been long suggested to be the process through which autoantigens, and subsequently autoantibodies, are formed. This hypothesis is summarised in

Figure 1.9.

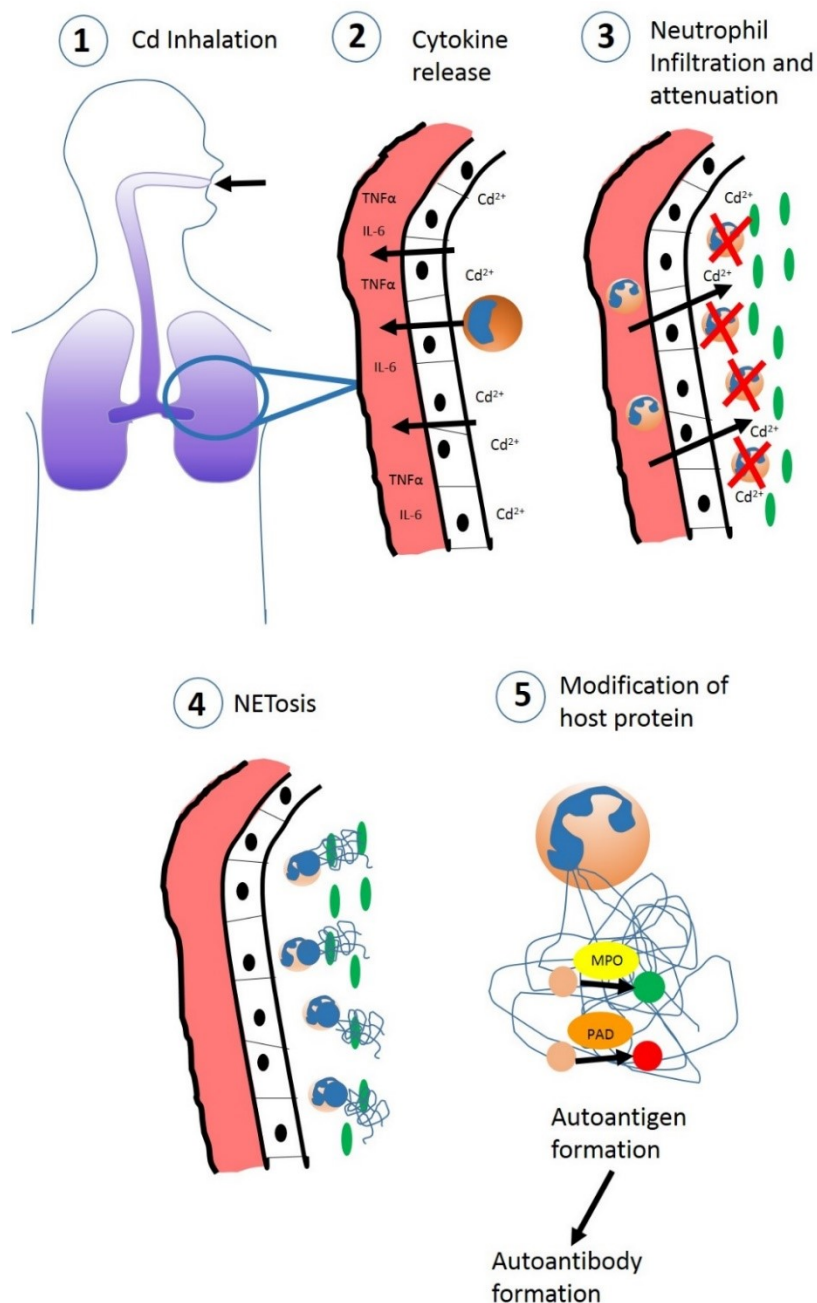


Figure 1-9 A hypothesis for how Cadmium may result in RA.

1) Cadmium is inhaled into the lungs. 2) Here, cadmium induces proinflammatory cytokine release from epithelial cells and alveolar macrophages. Neutrophils are drawn to the pulmonary tissue where cadmium neutralises activity, resulting in 4) NETosis as a last resort to pathogen clearance. 5) The enzymes involved in NETosis activation indirectly modify host proteins, facilitating autoantigen formation, and subsequent opportunity for B-cells to generate autoantibodies to these modified proteins.

1.6 Introduction summary and hypothesis

Cadmium is a dangerous element with many toxic effects in the body and has recently been suggested as a causative agent in RA. This would explain the major risk factor of smoking in RA development. Therefore, it is hypothesized that chronic cadmium inhalation causes prolonged inflammation in the lungs, which results in neutrophil dysfunction, where essential functions are hindered. This hindrance results in ineffective clearance of pathogens in the lungs, further causing inflammation. Ultimately, the diminished functional capacity causes NETosis to be a last-resort antimicrobial process, resulting in autoantigen formation. This occurs through activation of MPOs and PADs, which alter amino acid residues on structural proteins. Bronchiectasis is a risk factor for RA development through similar mechanisms, with the double hit hypothesis sharing similar steps in autoantigen formation. These autoantigens then trigger autoantibody formation towards structural proteins, of which similar-binding epitopes are generated. These epitopes then trigger a “second hit” in the joint, resulting in RA development, an autoimmune disease of poor quality of life in patients, for which there is no cure.

1.7 Aims of this thesis

This thesis aims to:

- Further investigate the impact of cadmium exposure on neutrophil biology, including respiratory burst and phagocytosis
- Examine if cadmium is capable of inducing NETosis in neutrophils
- Explore serum biomarkers in RA patients with either bronchiectasis or a history of cadmium exposure that may yield clues to mechanisms of autoantigen formation.

2 Materials and methods

2.1 General lab techniques

2.1.1 Protein sample quantification

Sample protein concentrations were determined employing several methods. First, a Denovix DS-11 series spectrophotometer/fluorometer was used to measure sample protein concentrations based on absorbance at 280 nm wavelength of ultraviolet light.

Alternatively, a bicinchoninic acid assay was performed using a Pierce™ BCA Protein Assay Kit (Pierce, UK). Serial dilutions of 2mg/ml of Bovine serum albumin were prepared in a 96 well microplate to generate a standard curve. Samples were diluted 1:10, 1:20 and 1:50 in double-distilled H₂O and added to the microplate at a volume of 25µl per well. A 200µl aliquot of BCA working reagent was then added to each well and mixed with the sample. Plates were then sealed and incubated at 37°C for 30 minutes and then read on an Optima Fluostar microplate reader (BMG labtech, UK). Absorbance was measured at 550nm excitation for each well and a standard curve based on absorbance values from the bovine serum albumin (BSA) serial dilutions was generated. The linear portion of this curve was then used to extrapolate sample protein concentration.

2.1.2 Western blotting

2.1.2.1 Gel loading

Test samples had their protein concentration measured in order to ensure equal protein loading of all samples.. Samples were mixed with sample buffer (Bio-Rad, UK) containing 84mM dithiothreitol (Sigma, UK.) Samples were heated to 90°C for 5 min to denature proteins. A precast 15-well, 8-16% acrylamide SDS-

PAGE tris-glycine gradient gel was inserted into a Bio-rad gel tank and filled with 500ml of tris/glycine/SDS running buffer (Bio-Rad, UK). Each gel was loaded with 3µl of precision plus all blue protein standards™ (Bio-Rad, UK) molecular weight markers. .Then 15µl of each denatured sample was added to each lane of the loading gel and run at constant voltage, initially at 70V for 20 min, followed by 120V for 100 min.

2.1.2.2 Transfer

Gel transfers were performed using the Bio-Rad turboblot system (Bio-Rad, UK). Once gel runs were completed, the gel was removed from the casing and placed between the bottom and top layer of a Trans-Blot® Turbo™ mini polyvinylidene fluoride membrane transfer Pack. The moist filter paper/gel/membrane/filter paper sandwich was then placed inside one of the two cassettes in a turboblot system, sealed, and subsequently inserted back into the equipment. The proteins were ran on a mini-TGX program, consisting of a 3 min, 2.5A 25V transfer.

2.1.2.3 Antibody staining

On completion of the transfer, , the membrane was removed from the cassette and was washed in phosphate-buffered saline (PBS) for 5 min. The membrane was then blocked with Pierce protein-free blocking buffer (Pierce, UK) for 1 h at room temperature. Primary antibodies were added at optimal concentrations diluted in protein-free blocking buffer and membranes were incubated overnight at 4°C with gentle rocking. The following day, membranes were washed in phosphate buffered saline with 0.1% v/v Tween-20 (PBST) for 3x 5 min. The

appropriate secondary antibodies were then added and incubated at room temperature in the dark for 1h. A final wash step of 3 x 5 min washes in PBST was performed before imaging the blot.

2.1.2.4 Blot imaging

Blots were scanned using near-infrared fluorescence using the **Odyssey** CLx Imager system (Li-Cor Biosystems, UK). Blots were kept moist before a scanning run was performed. Image analysis and quantification was performed using the Li-Cor image studio lite software (version 5.2). Fluorescence intensity for each band was measured via densitometry analysis.

2.2 Cell culture

2.2.1 General plastic consumables and chemical reagents

All consumable plastics used for experimental purposes in cell culture were purchased from Greiner Bio-One, Austria. This includes sterile microplates, culture flasks, centrifuge and microcentrifuge tubes, serological pipettes and pipette tips. Cell culture grade, sterile Hanks Buffered Salt Solution without calcium and magnesium ions (HBSS) was purchased from Gibco (Thermo Fisher Scientific, UK).

2.2.2 Cell culture conditions

All cells were incubated at 37°C with 5% v/v CO₂, with atmospheric levels of oxygen. Cells were cultured with RPMI-1640 or and supplemented with 10% v/v foetal bovine serum (Gibco, UK) and 1% v/v Penicillin/Streptomycin (10,000

U/ml) (Gibco, UK) and 1% v/v L-Glutamine (200mM). Cells were passaged every 7-14 days. During passaging, cells were pelleted via centrifugation at 300 RCF for 5 min. After discarding the supernatant, the pellet was resuspended in fresh culture medium and transferred to a fresh flask.

2.2.3 Cell lines

Initially, neutrophil experiments were first conducted and optimised employing a HL60 cancerous promyeloblast cell line (ATCC, USA). This cell line was differentiated into a neutrophil-like phenotype via treatment with 1 μ M 1-All-Trans-Retinoic Acid (Sigma, UK). This process required 1x10⁵ HL60 cells to be centrifuged at 500 g for 5 minutes, followed by resuspension of the pellet with 10ml differentiation media. This consisted of RPMI-1640 (Gibco, UK), 1 μ M 1-ATRA, 1.3% v/v DMSO and 5% v/v FBS. After 3-5 days of incubation, confirmation of differentiation was confirmed via Diffquik stain.

2.3 Primary neutrophils

2.3.1 Volunteer selection for blood donation

Blood was obtained from volunteers who provided written informed consent in accordance with University of Exeter Medical School research ethics committee code May18/B/152 Δ 1. Venous blood samples were collected from the median cubital vein with butterfly 21-gauge needles (BD, Oxford, UK) and 10ml capacity EDTA-coated vacutainer tubes (BD, Oxford, UK). Each volunteer donated up to 50ml of venous blood no more than three times, at least two weeks apart before being excluded from providing any further samples for 3 months.

2.3.2 Primary human neutrophil isolation procedure

Five ml aliquots of venous blood were layered carefully on top of 5ml of Polymorphprep (Abbott, UK) in 15ml centrifuge tubes. The tubes were then centrifuged 650 RCF for 30 mins at 18-22 °C. The translucent PMN layer was harvested from the tubes and osmolarity of cell suspension was restored by a v:v addition of 0.45% w/v NaCl. The cell suspension was then centrifuged at 18-22 °C at 400 RCF for 10 min and resuspended in 5ml isotonic NH₄Cl₂.

Following a 5 min incubation at 37°C to allow red blood cell lysis to occur, the tube was centrifuged again at 18-22 °C at 400 RCF for 10 min and resuspended in HBSS without calcium or magnesium ions.

2.3.3 Determination of Neutrophil population cell purity

2.3.3.1 Romanowski Staining

To assess neutrophil purity, 50µl aliquots of cell were spun onto a glass slides via a cytospin centrifuge. The adherent cells were air dried by shaking and fixed in 100% methanol for 10 secs, then stained rapidly stained with Eosin G for 10 secs, followed by a Methylene blue stain for 10 seconds. The slides were washed in ddH₂O and then imaged via light microscopy. The purity of the cells was assessed by nuclear staining and morphology.

2.3.3.2 Flow Cytometric analysis of surface marker expression

Purified isolated cells were counted using the Bio-Rad TC20 automated cell counter. Next 50,000 cells were transferred to fresh 1.5ml reaction tubes and resuspended in 100µl HBSS with 2% w/v BSA. Neutrophils were probed with a panel of antibodies against the following targets: CD11b, CD16, CD62L and CD66. Cells were incubated with 1:50 dilution for each antibody for 30

minutes in the dark on ice. Further details on the selected antibodies can be found in **Table 2.1**. Following incubation, excess antibody were removed by washing the cells in 900µl of ice-cold HBSS with 2% w/v BSA was added and centrifuged at 300RCF for 5 mins. Cells were fixed by resuspension in 4% w/v Paraformaldehyde and kept at 4°C in the dark until analysis. Flow cytometry was performed on a Guava Easycyte Mini2 (Millipore,UK). The process of gating to assess neutrophil purity can be seen in **Figure 2.1**.

Antibody Target	Fluorophore	Product Code	Excitation	Emission	Supplier
CD11b	PerCP-eFluor710	15589076	490	710	eBioscience
CD16	AlexaFluor488	15347394	495	519	Invitrogen
CD62L	PE	11519242	480	575	Invitrogen
CD66	APC	15517966	650	660	eBioscience

Table 2-1 Fluorescent antibodies used for flow cytometry

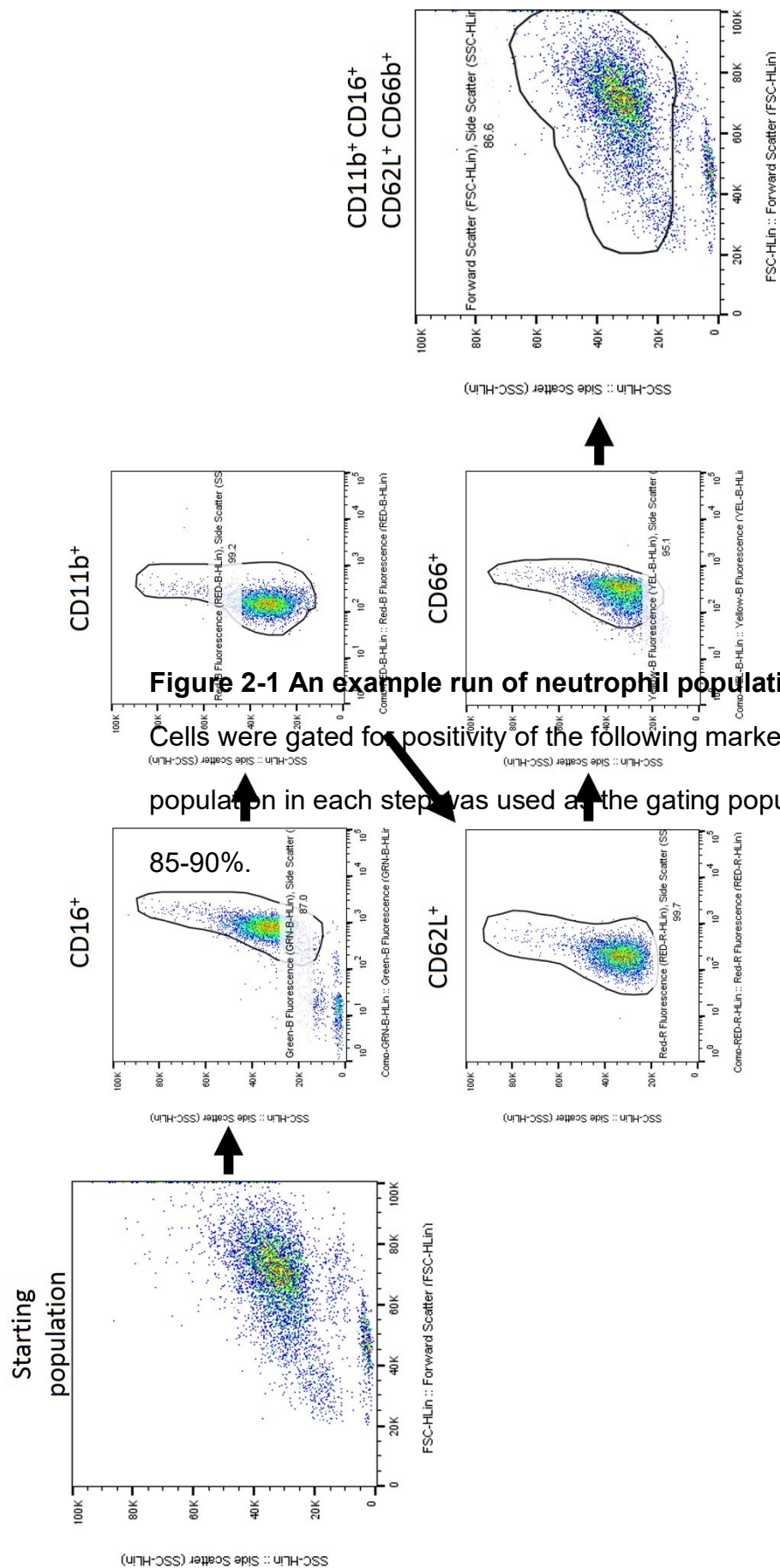


Figure 2-1 An example run of neutrophil population gating to assess Polymorphonuclear Leukocytes (PMNs). Cells were gated for positivity of the following markers in subsequent steps: CD16, CD62L, CD66, and CD11b. The population in each step was used as the gating population for the next step. Neutrophils were identified as CD16⁺ CD62L⁺ CD66⁺ CD11b⁺.

2.3.4 Flow cytometry

2.3.4.1 Samples used for flow cytometry

A number of fluorimetric assays were established to assess neutrophil function upon treatment with various test reagents as detailed below. Neutrophils were treated with various concentrations of cadmium chloride (CdCl_2): 1 μM , 3 μM , 10 μM , 30 μM and 100 μM . Additionally serum from RA patients of varying disease severity or healthy control serum was added to cells at a 1:50 dilution. Finally, unmodified or citrullinated and carbamylated Fc fragment of immunoglobulin G (here to referred as mod-Fc) was added to cells at 100ng/ml.

2.3.4.2 Measurement of Neutrophil Respiratory Burst

To examine if various molecules and compounds were capable of inducing the respiratory burst in neutrophils, the fluorescent dye dihydrorhodamine 123 (DHR123 - Invitrogen, UK) was used. DHR123 works by emitting a fluorescent green colour in the presence of reactive oxygen species. This is an oxidation reaction whereby DHR123 is converted to cationic Rhodamine 123 in the presence of hydrogen peroxide, an intermediate product of ROS generation in neutrophils. Aliquots of 5×10^4 cells in HBSS were incubated with 1 μM DHR123 and the treatments listed above. HBSS alone or containing 100nM of PMA acted as a negative or positive control, respectively. Samples were incubated at 37°C for 30 minutes and the reaction was terminated by placing the sample tubes on ice for 10 minutes. Cellular levels of green fluorescence at 488 nm was quantified using a Guava EasyCyte Mini2 flow cytometer (Millipore, UK).

2.3.4.3 Measurement of Neutrophil internal calcium flux

Human neutrophils ($1-5 \times 10^6$ cells/ml) were loaded with the fluorometric calcium indicator fluo-4 AM (Invitrogen, UK) at a concentration of $2.5 \mu\text{M}$ in HBSS and incubated for 45 minutes at 37°C . After incubation, cells were centrifuged at 300 RCF and resuspended in fresh HBSS. A cell count was performed and 5×10^4 cells were transferred into a 1.5ml reaction tube and resuspended to a volume of $500 \mu\text{L}$ in HBSS. The experimental fluorometric procedure occurred in three sequential steps in each sample tube, to measure basal, treatment and maximum calcium flux. During these steps, green fluorescence emission from the Fluo-4-AM dye detecting calcium release was measured. First, $100 \mu\text{L}$ of cell suspension was ran through the flow cytometer and the MFI measured, then the run was terminated. Second, $100 \mu\text{L}$ of treatment compounds of interest were added at 5x concentration to the cells. In the case of the patient serum, a 1:10 dilution of serum was prepared and immediately added to each experimental tube. The final experimental dilution was a 1:50 dilution and fluorescence was immediately recorded upon addition of serum. HBSS was added as a negative control, or 15 ng/ml of interleukin-8 (final concentration of 3 ng/ml - Peprotech, UK) to act as a positive control. Once again, $100 \mu\text{L}$ of cell suspension with the added treatments was ran through the machine and then terminated. Third, $100 \mu\text{L}$ of $1 \mu\text{M}$ ionomycin (Sigma, UK) was added to the tube and ran through the machine. Median fluorescence intensity for each step was measured and compared between treatment groups.

2.3.4.4 Measurement of Neutrophil phagocytic capacity

Firstly, pHrodo Green fluorescently labelled heat killed *E. Coli* (BioParticles – invitrogen, UK) were opsonised in autologous donor serum at a concentration of

10µg/ml at 37°C for 30 minutes. 5x10⁴ neutrophils were then incubated with the opsonised particles at a final concentration of 1µg/ml along with a series of concentrations of CdCl₂ (1- 100µM).

2.3.4.5 Determination of cytotoxicity of cadmium chloride

An Alexafluor 488 annexin V/ dead cell apoptosis kit (Invitrogen, UK) was used to examine any potential cytotoxic effects of cadmium exposure to neutrophils. Purified Neutrophils were diluted in HBSS supplemented with 10% v/v autologous donor serum, then added to 1.5ml reaction tubes at 1x10⁵ cells per tube. Cells were treated with 1,3,10, 30 and 100µM of CdCl₂ for 3, 12 and 24 hours. HBSS and 1µM H₂O₂ were used as negative and positive controls, respectively. After each of the respective incubations, cells were centrifuged at 300RCF for 5 minutes, and washed once in ice-cold fresh HBSS^{-/-}. Cell pellets were resuspended in annexin V binding buffer (as supplied by the assay kit), 1µM of propidium iodide, and a 1/40 dilution of annexin V. Cells were incubated at room temperature for 15 minutes, then placed on ice and analysed by flow cytometry immediately.

2.3.4.6 Analysis

For all flow cytometry experiments conducted, flow cytometry files were exported from the native Incyte software and analysed using FlowJo (TreeStar Inc, UK). Cell populations concluded to be CD11b⁺CD16⁺CD62L⁺CD66⁺ were determined to be neutrophils and gated for analytical purposes. For further analysis of fluorescence values, the median values in relevant channels were calculated and used in statistical analysis.

2.3.5 Induction and measurement of NETosis

2.3.5.1 Live Cell Imaging

In order to visualise primary neutrophils undergoing NETosis, cells were first incubated with 1 μ M of the cytoplasmic stain Dil for 1 hour at 37°C. Purified neutrophils were resuspended in HBSS. For 1-ATRA differentiated HL60s were suspended in serum-free, penicillin/streptomycin free RPMI-1640 for. Both cell types were plated into wells of 96 well plates at a density of 5×10^4 cells per well in a 100 μ l final volume. After seeding, the plate was placed inside the JuLi stage live cell imaging system (NanoEntek, Korea), situated inside an incubator. The plate was incubated at 37°C for 1 hour to allow cells to adhere to the bottom of the plate. Once adhered, cells were treated with 10x concentration of PMA, SYTOX Green (Invitrogen, UK) and Hoescht 33342 stain (Invitrogen, UK). SYTOX Green is a non-permeabilizing DNA dye that fluoresces green in the presence of DNA. The combination of the three dyes used allows visualisation of cell cytoplasm whilst discerning between externalised DNA and live cells that had not yet undergone NETosis. Final concentrations in each well were 50nM PMA, 500nM SYTOX Green and 1 μ g/ml of Hoescht. Images of each well were taken every 5 minutes for 4 hours, capturing blue, green and red fluorescence. Images were analysed using JuLi EDIT software (Nanoentek, Korea.)

2.3.5.2 SYTOX Green Spectrofluometric Assay

Aliquots of neutrophils (1×10^5 cells/ well in 100 μ l in HBSS) were seeded onto a 96 well plate and allowed to adhere to the bottom of the wells for 1h at 37°C. . Next equal volumes of 2x concentration of treatment compounds were added, to provide final concentrations in each well of 50nM PMA, 50, 100, 500 and 1000ng/ml, LPS derived from *Pseudomonas aeruginosa* (Sigma, UK) and 1,

2.5, 5, 25 and 50 μ M of CdCl₂. Additionally, in some experiments, serum from RA patients or healthy donor was added at 1:10 dilutions. Plates were incubated at 37°C for 4 and 24 hours followed by subsequent addition of 1 μ M SYTOX green. The fluorescence was then measured on an Optima PHERAstar plate reader (BMG Labtech, UK) at 485nm excitation and 525nm emission wavelengths and RFUs were recorded.

2.3.5.3 Immunofluorescence

Aliquots of 1x10⁵ Neutrophils were incubated into 8-well Nunc Lab- Tek II chamber slides and left to adhere for 1 hour at 37°C. 100nM PMA (final concentration) was then added to induce neutrophils NETosis over 4 hours at 37°C . Given the fragility of NETs, after treatment, 8% w/v paraformaldehyde was added to each well to fix cells. Supernatants were then aspirated and slides were washed in 250 μ l of PBS three times. Cells were blocked in 10% v/v goat serum for 1 hour at room temperature. The slides were washed in 3x250 μ l of PBS and the cells incubated with a 1:100 dilution of rabbit anti-citrullinated histone H3 antibody (Abcam, UK) and mouse anti-myeloperoxidase (Abcam, UK) for 1h at room temperature. Slides were washed three times in PBS and incubated with AlexaFluor 594-conjugated and AlexaFluor488-conjugated goat anti-mouse and anti-rabbit secondary antibodies, respectively (Life Technologies, UK) for 1h at room temperature in the dark. Slides were washed 3 times in PBS and then the well divider on the slide was removed. Slides were then mounted with Prolong Gold Antifade Reagent with DAPI (Invitrogen, UK) and sealed. Slides were then visualised on an EVOS fluorescence microscope at 10x and 40x magnification (Thermo Fisher Scientific, UK). The native microscopy software on the microscope was used to generate overlays for double and triple stained images.

2.4 Immunoglobulin G and patient serum biochemistry

2.4.1 Patient population.

Patient samples were obtained through a multi-centre ethics IRAS 12324, with patient recruitment taking place between May 2012 and May 2013. Sera from patients and controls were selected from the BRACRA (bronchiectasis, asthma, control, rheumatoid arthritis) study: a prospective, multicentre, case–control, observational study, conducted to determine the relationship between bronchiectasis and RA development and the overall study design, the assays used for the determination of RF and anti-CCP levels and approval has been previously reported (320). All the RA patients fulfilled the ACR 2010 classification criteria for RA and the definition of a negative, low positive and high positive RF and anti-CCP is as per the above classification criteria (199). An additional cohort of 220 serum samples was gathered from RA patients in the south west. The samples used in this study were gathered as part of a project as part of project IRAS ID 194833, approved by South West Regional Ethical Committee (UK).

2.4.2 Sample preparation for mass spectrometric analysis

Equal serum protein loads as determined by nanodrop spectrometry were separated on 8-16% SDS-PAGE gradient gels (Bio-Rad, UK). A protein band identified by immunoblotting with anti-human citrulline antibody (Abcam ab100932, Abcam, UK), contained numerous citrullinated proteins in the region of 37-50 kilodaltons (KDa).

2.4.3 Mass spectrometry

2.4.3.1 Band digestion

The bands were excised from Bio-safe Coomassie (Bio-Rad,UK) stained gradient gels and subjected to in-gel tryptic digestion using a DigestPro automated digestion unit (Intavis Ltd, UK).

2.4.3.2 Mass spectrometry sample analysis

The resulting peptides were fractionated using an Ultimate 3000 nanoHPLC system. Tandem mass spectra were acquired using an LTQ- Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific,UK) and operated in data-dependent acquisition mode. .

The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific, UK) and searched against the UniProt Human database (131351 entries) using the SEQUEST algorithm. Search criteria routinely included carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of methionine as a variable modification. In addition, citrullination (+0.984Da) at Arg, AsnN and GlnQ and carbamylation (+43.006Da) at Lys, Met, ArgR, Ser, Thr and TyrY, were included as variable modifications in two separate searches. Only peptides where citrullination at Arg and carbamylation at Lys were ranked 1 in the respective searches (indicating that those residues were the most likely sites of modification) were considered.

2.4.3.3 Protein Modelling

The UCSF Chimera protein viewing software (<http://www.rbvi.ucsf.edu/chimera/>, USA) was used to annotate the amino acid modifications of interest to highlight the citrullinated and carbamylated sites on the proteins of interest.

2.4.4 Immunoglobulin G purification

Immunoglobulin G was isolated from commercially available human serum (Sigma, UK) and purified on a Hitrap Protein G column on an AKTA FPLC purifier (GE Healthcare, USA). Samples were diluted 1:5 in 20mM sodium phosphate buffer pH 7.0 and eluted off the column by 0.1M Glycine-HCl pH 2.7. Fractions collected during the elution window are classified as pure IgG.

2.4.5 Fc fragment preparation

2.4.5.1 Immunoglobulin G papain digestion and Fc fragment purification

2 mg/ml of purified IgG in Sodium Phosphate Buffer (pH 7.4) was treated at 1:20 Enzyme:Protein ratio (w/w) with Papain (Sigma, UK) in digestion buffer. Digestion buffer consisted of 0.02M EDTA and 0.02M L-Cysteine in phosphate buffered saline (PBS). The reaction was incubated at 37°C for 2 hours and terminated with 0.3M Iodoacetamide dissolved in PBS. Samples were buffer-exchanged using vivaspin 500 MWCO protein concentrators into 20mM sodium phosphate buffer pH 7.0. Samples were then rerun on the AKTA purifier using the above protocol. Successful papain cleavage was confirmed via SDS-PAGE using an 8-16% tris-glycine gradient gel.

2.4.5.2 *Post-translational modifications of Fc fragment*

To citrullinate Fc fragment, 1 unit of Peptidyl Arginine Deiminase from rabbit skeletal muscle (Sigma, UK) was used per 250µg of protein. The reaction was buffered by a solution of 0.1M Tris/HCL, pH7.6, 10mM CaCl₂, 5mM DTT.

Reactions were incubated overnight at 37°C.

For carbamylation, Fc fragment isolate was diluted to a concentration of 2mg/ml and incubated with 0.1M Potassium Cyanate in 0.15M sodium phosphate buffer (pH 7.4). The reaction was then incubated overnight at 37°C for 24h and then buffer exchanged with sodium phosphate buffer to remove unreacted cyanate.

Considering double modification of Fc fragment would require a chemical and enzymatic approach, it was deemed suitable to enzymatically and then chemically modify Fc fragment, in order to preserve potential substrate sites on the protein. After the initial enzymatic reaction, deimination buffer was removed by buffer exchange before resuspension in cyanate reaction buffer.

2.4.6 Fc fragment ELISA

In order to measure serum antibody levels to the unmodified and modified Fc fragment of IgG in the sera of each test cohort, an in-house capture ELISA was designed. 96-well high binding microplates (Greiner Bio-One, Austria) were coated with a 5µg/ml dilution of either untreated Fc fragment (UT-Fc), or double modified Fc fragment (mod-Fc). UT-Fc was added to rows A-D of the microplate, and mod-Fc was added to rows E-H. The plate was then sealed and incubated overnight at 4°C to allow the antigens to adhere to the plate.

Following incubation, the plate was washed 4x with 200µl of 0.1% v/v PBST and remaining binding sites were subsequently blocked with 200µl of 5% w/v BSA in

0.1% v/v PBST for 30 minutes at 37°C. Wells were washed again 4 times in 0.1% v/v PBST. At this point, the plate was divided into four quadrants, detailed in **Table 2.2**. Next, 100µl of a 1:50 dilution of cohort sera in 0.1% v/v PBST was added to the top-left corner of each quadrant and added to each of the four quadrants, resulting in 24 samples being added to the plate four times each. PBST was used as a blank, occupying the bottom-right well of each quadrant. The plate was sealed and incubated for 2 hours at 37°C. The wells were washed 4x in PBST once more, and an anti-human IgM or IgA HRP-conjugated secondary antibody was added to the plate in **Table 2.2** at a 1:2000 dilution in PBST. Following a 1h incubation at 37°C, the plate was washed as mentioned above, and 100µl of SureBlue™ TMB 1-Component Microwell Peroxidase Substrate (KPL, UK) was added to each well on the plate and left to incubate in the dark for 15 minutes. This reaction was then terminated by adding 50µl of 1M H₂SO₄ to each well. The optical density of each sample was measured on a BMG Labtech Fluorostar plate reader at 450nm excitation.

2.5 Statistical analysis

All statistical analysis used throughout this thesis was performed using the Graphpad Prism software (version 8-10). To compare two groups, a Mann-Whitney test was performed. Analysis between multiple groups used either a Kruskal-Wallis test with Dunn's post hoc test, or a Within Sample two-way ANOVA with Dunnett's or Holm-Sidak's multiple comparison post hoc test as appropriate, unless stated otherwise. Statistical significance was classified as a p value of less than 0.05.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	UT-Fc IgM response				1	2	UT-Fc IgA response			
B												
C												
D												
E	1	2	Mod-Fc IgM response				1	2	Mod-Fc IgA response			
F												
G												
H												

Table 2-2 Template for Fc fragment ELISA.

Either unmodified or mod-modified Fc fragment was added to a 96 well plate.

Unmodified Fc fragment was added to the top four rows (rows A-D) and the modified Fc fragment was added to the bottom four rows (E-F). After blocking, serum samples were added to same well in each quadrant (i.e. sample 1 in A1, A7, E1, E7, sample 2 in A2, A8, B2, B8 and so forth). After serum sample incubation and subsequent wash step, secondary antibody was added.

Secondary antibody was added as follows: anti-human IgM was added to columns 1-6, and anti-human IgA was added to columns 7-12. After incubation, substrate was added and the OD_{450nm} was measured. This results in the quantification of the IgM and IgA response to both unmodified and modified Fc fragment.

3 The effect of cadmium on neutrophil biology

3.1 Introduction

As outlined in the introduction, research into the effect of cadmium on neutrophil biology remains rather limited, with no substantial investigations having been explored since the 1980's. The link between cadmium and RA recently suggested by this research group indicates that the lungs are the initiating site for RA development in exposed individuals (321). In most pulmonary diseases, heavy neutrophil involvement is a key driver of pathology. Coupled with the recent evidence of cigarette smoking inducing NETosis, the role of cadmium on general neutrophil function may lead to clues as to how autoimmune-associated mechanisms may be triggered. The objectives of this chapter were to:

- Explore the effects of cadmium on general neutrophil physiology, and any resulting dysfunction which may link to RA pathology. The cellular functions examined were:
 - Cell viability
 - Oxidative burst
 - Phagocytic capability
 - Calcium flux
 - NETosis

It was theorized that cadmium would principally induce NETosis in neutrophils, highlighting this as the causative agent found in the work of Qiu *et al* (123).

Calcium flux was examined as the increase of cytosolic calcium levels is also an essential step in NET formation, which may hint at further clues to this relationship.

3.2 The effect of cadmium on cell viability

To investigate the effect of cadmium on neutrophil biology, the cytotoxic effects of cadmium on neutrophils was examined. Isolated neutrophils were suspended in HBSS^{-/-} supplemented with autologous donor serum and treated with a range of concentrations of cadmium chloride (1, 3, 10, 30 and 100 μ M CdCl₂) for 3, 12 and 24 hours, and cytotoxicity was determined by flow cytometry using annexin V-PI staining.

Early apoptosis is defined as cells binding only annexin V, whereas late apoptosis is defined as cells binding both annexin V and PI. Necrosis is classified as cells only testing positive for PI, though no necrosis was observed in these cells. Incidence of both early and late stage apoptosis was not significantly increased at any of the time points measured. Based on these data, it would suggest that cadmium is not toxic to healthy neutrophils (**Figure 3.1 and figure 3.2**).

Cadmium is known to oxidative stress in various cell types in the human body (33). Production of reactive oxygen species is an essential function of neutrophil innate immune biology (79-81). The production of ROS can also lead to damage in the surrounding tissues. To current knowledge, the effect of cadmium on ROS production in the context of neutrophil biology has not been examined before. Consequently an assay was established to measure intracellular ROS production using the fluorescent probe dihydrorhodamine 123 (DHR123). DHR123 is converted to rhodamine 123 in the presence of hydrogen peroxide, an intermediate molecule generated during ROS production. This reaction converts a non- fluorescent molecule to a green fluorescent molecule, allowing semi- quantification of ROS production in cells by fluorescent monitoring using flow cytometry.

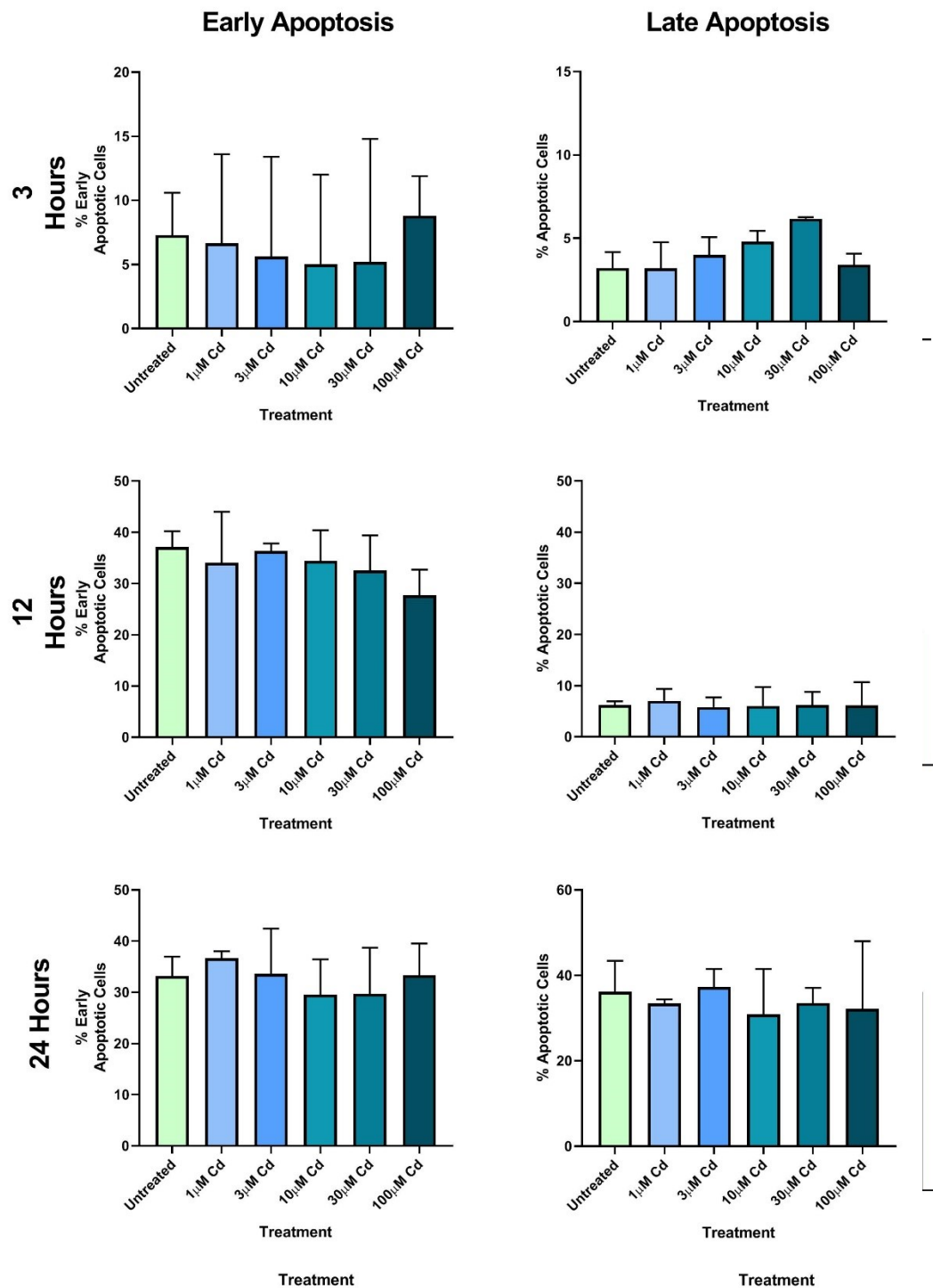


Figure 3-1 Cadmium chloride is not cytotoxic to freshly isolated neutrophils.

Cytotoxicity of cadmium chloride to neutrophils was used by annexin-V/PI staining over a 3, 12 and 24h timepoint. Gating was compared against untreated cells.

Statistical significance was tested by Kruskal-Wallis with Dunn's post-test. Data are presented as median % of cells in early/late apoptosis \pm IQR over 3 independent experiments using different donors.

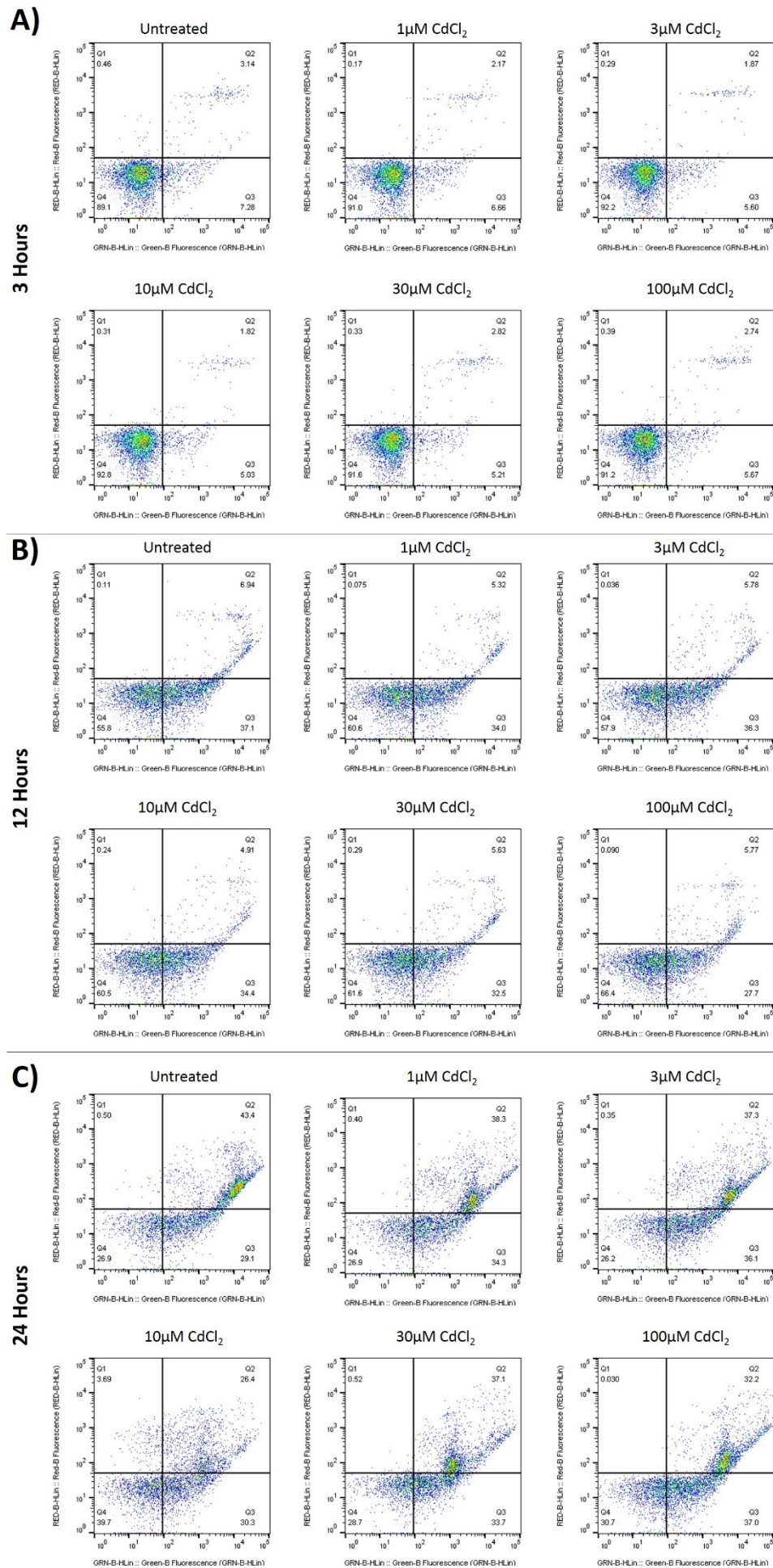


Figure 3-2 Representative scatter plot of annexin-V/PI staining to determine cell viability.

Scatter plots showing cell positivity for annexin V (x axis) against cell positivity for propidium iodide (y axis). Scatter plots for each treatment are shown at A) 3 hours, B) 12 hours and C) 24 hours. Positivity for each stain was calculated by gating the 99th percentile of single stain controls. This gating was then applied to all experimental samples.

Based on the method first developed by Chen and Junger, Isolated neutrophils were exposed to cadmium chloride for 30 minutes to test the acute response to cadmium treatment, compared to PMA which is a rapid inducer of the respiratory burst in neutrophils through PKC activation (322).

Cadmium chloride in the doses and time tested did not induce production of reactive oxygen species compared to above untreated cells (**Figure 3.3**). No statistical significance (Kruskal-Wallis test with Dunn's post test) in median MFI between unstimulated neutrophils and 1, 3, 10, 30 and 100 μ M CdCl₂ ($P>0.05$) was observed.

These results suggest that direct exposure of neutrophils *in vitro* to cadmium does not induce acute production of ROS.

An additional observation from the respiratory burst assay found that upon stimulation with PMA, morphological changes in neutrophils were observed, with cells increasing in size as shown by increase in FSC. In contrast, neutrophil morphology remained unchanged when exposed to various doses of cadmium chloride (**Figure 3.4**). There was no statistically significant difference in cell morphology based on neutrophil volume upon treatment with any of the experimental doses of cadmium ($P>0.05$). PMA induced significant ($P= 0.059$) increases in neutrophil size (**Figure 3.5**). Taken together, this would suggest that cadmium does not induce changes that causes potent activation of these cells.

Cadmium appears not to induce an acute respiratory burst directly. Next, whether cadmium played a role in attenuating the respiratory burst in the presence of a ROS stimulant (PMA) was investigated.

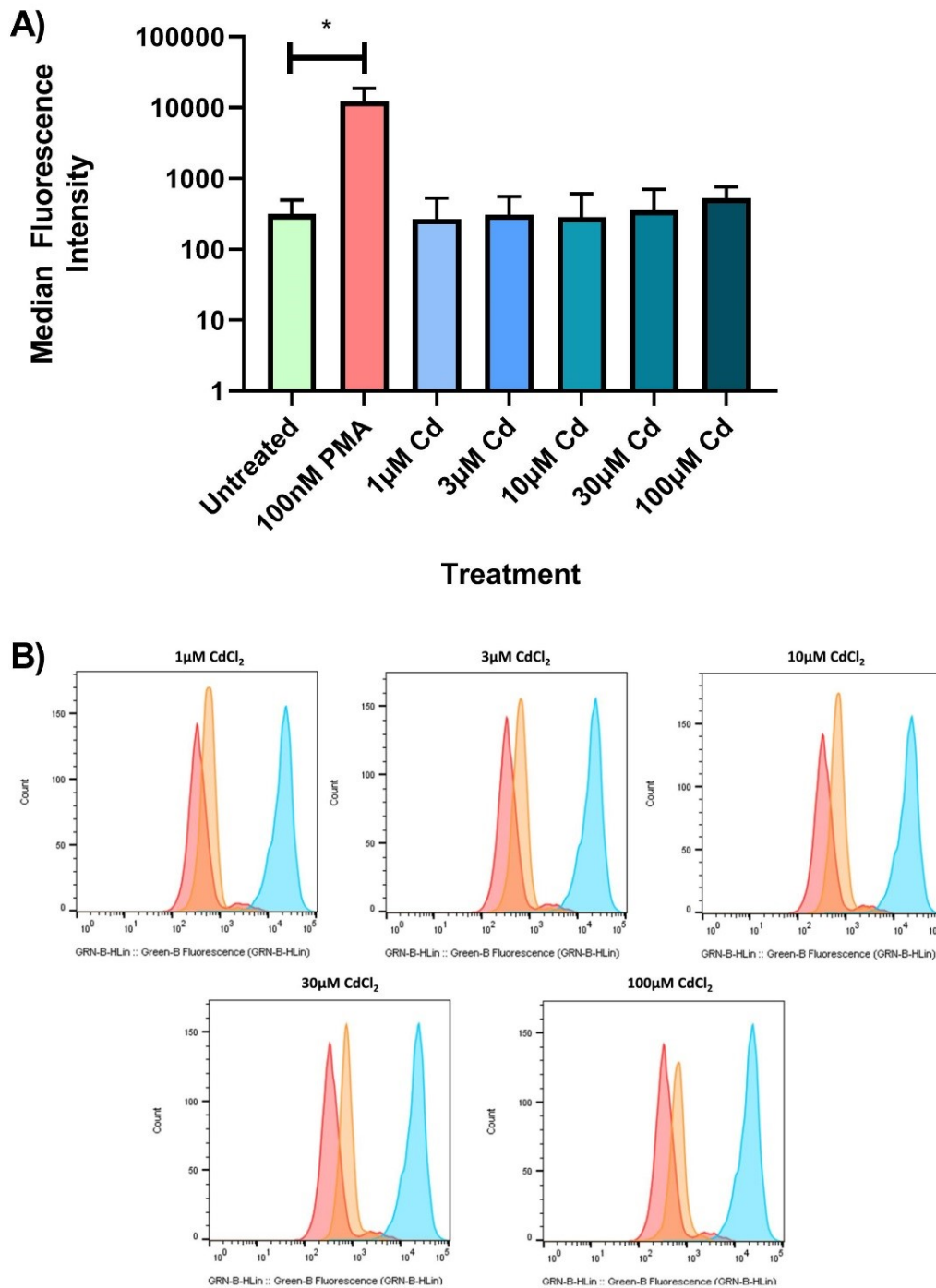


Figure 3-3 Cadmium does not induce the respiratory burst in primary neutrophils.

A) Neutrophils were incubated with dihydrorhodamine 123 and 1, 3, 10, 30 and 100µM CdCl₂ for 30 minutes after which green fluorescence was measured at 488 nm. Statistical analysis using a Kruskal-Wallis test with Dunn's post-test found no statistical significance between cadmium

treatment and MFI compared to the untreated HBSS control, demonstrating no effect of cadmium on ROS production in neutrophils. Data are shown as median MFI \pm IQR as a result of 5 independent experiments with different donors. * = $P < 0.05$. B) Flow histograms displaying a representative DHR run. Red peaks indicate the fluorescence recorded from HBSS treatment. Blue peaks indicate the fluorescence recorded from PMA treatment. Yellow peaks indicate the fluorescence recorded from treatment with the concentration of cadmium chloride listed above each histogram.

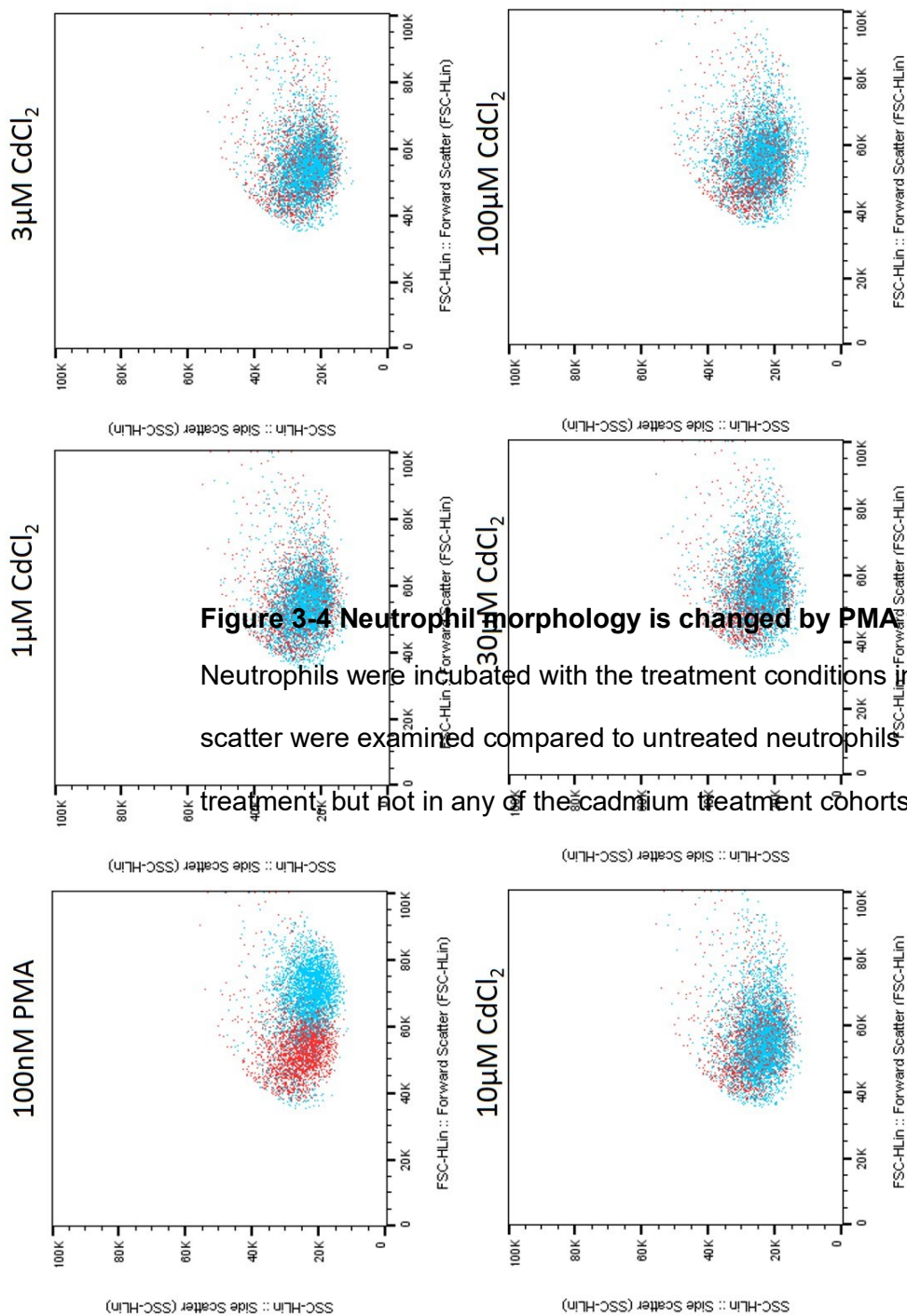


Figure 3-4 Neutrophil morphology is changed by PMA treatment, but not cadmium treatment. Neutrophils were incubated with the treatment conditions indicated in each panel above indicating the treatment. Changes in cell morphology were examined by flow cytometry. Forward scatter (FSC-HLin) and side scatter (SSC-HLin) were examined compared to untreated neutrophils (indicated in red). Increases in the side scatter were observed in the PMA treatment, but not in any of the cadmium treatment cohorts. Dot plots representative of five independent experiments.

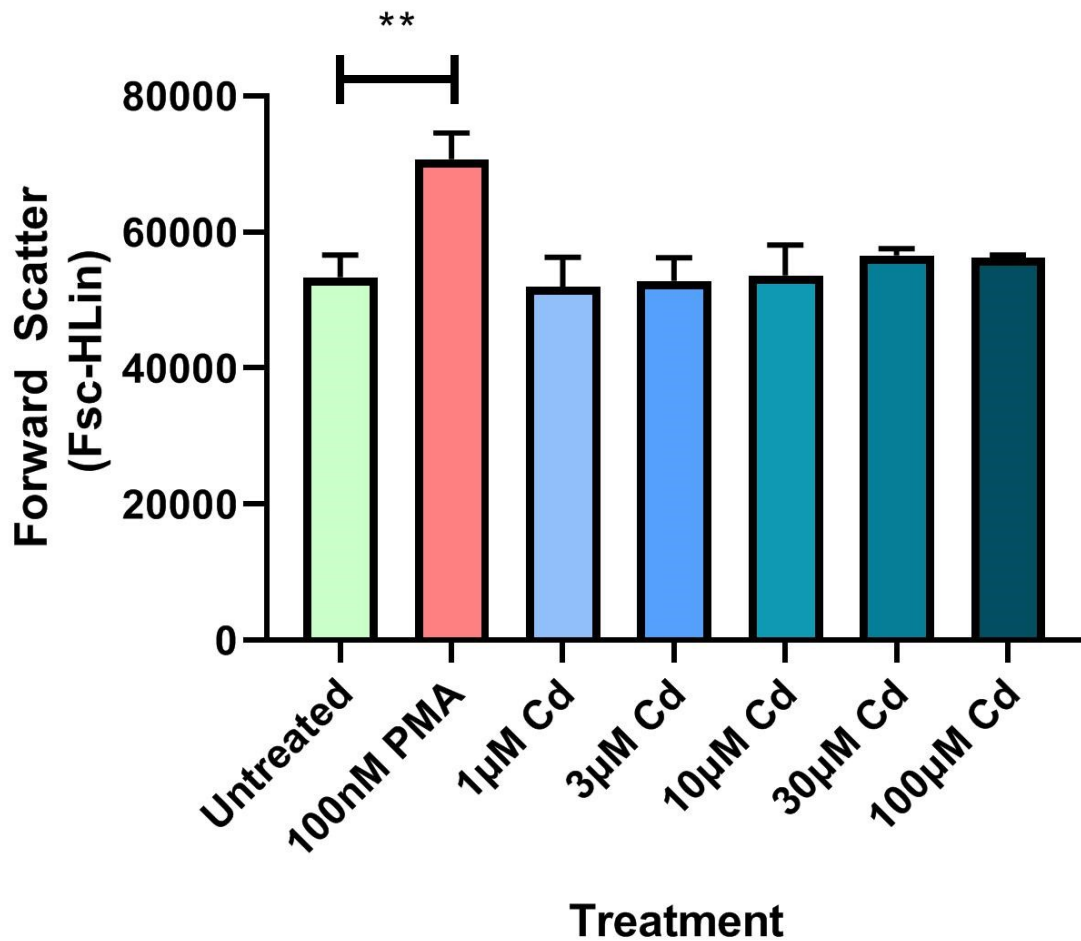


Figure 3-5: PMA induces significant increases in cell size, but not cadmium.

PMA statistically significantly increased cell size by means of FSC measurement. Cadmium did not induce any change in cell size. Statistical analysis used a Friedman's test with Dunn's post test. Data are presented as median MFI \pm IQR over five independent experiments. ** = $p < 0.01$

As described above, neutrophils were treated with 1, 3, 10, 30 and 100 μ M CdCl₂, with 100nM PMA and DHR123 and compared to PMA-only stimulated neutrophils (**Figure 3.6**).

A single 3 μ M cadmium dose increased production of ROS in neutrophils treated with 100nM PMA ($P = 0.0019$). All other concentrations of cadmium used showed no statistically significant difference.

3.3 Cadmium does not affect the phagocytic capacity of cells

Phagocytosis is a major function of neutrophils, and along with ROS production, these two mechanisms are integral to the innate immune clearance of pathogens.

As cadmium does not directly induce ROS production under the concentrations used, the effect of cadmium on phagocytic capacity was examined by quantifying the uptake of fluorescently labelled heat-killed bacteria with and without opsonisation.

Initial experiments found poor uptake of particles when incubated directly with the neutrophils. Subsequent experiments employed bioparticles that had been opsonised with autologous serum from donor subjects for 30 minutes. After opsonisation, these particles were directly added to 5×10^4 neutrophils and after 2 hours, cell fluorescence in the cells was measured to quantify uptake of bioparticles. Fluorescence peaks were normalized to untreated cells. A 30 minute pre-incubation with cadmium did not significantly alter the uptake of bioparticles over a subsequent 2 hour period at any of the concentrations tested (**Figure 3.7**).

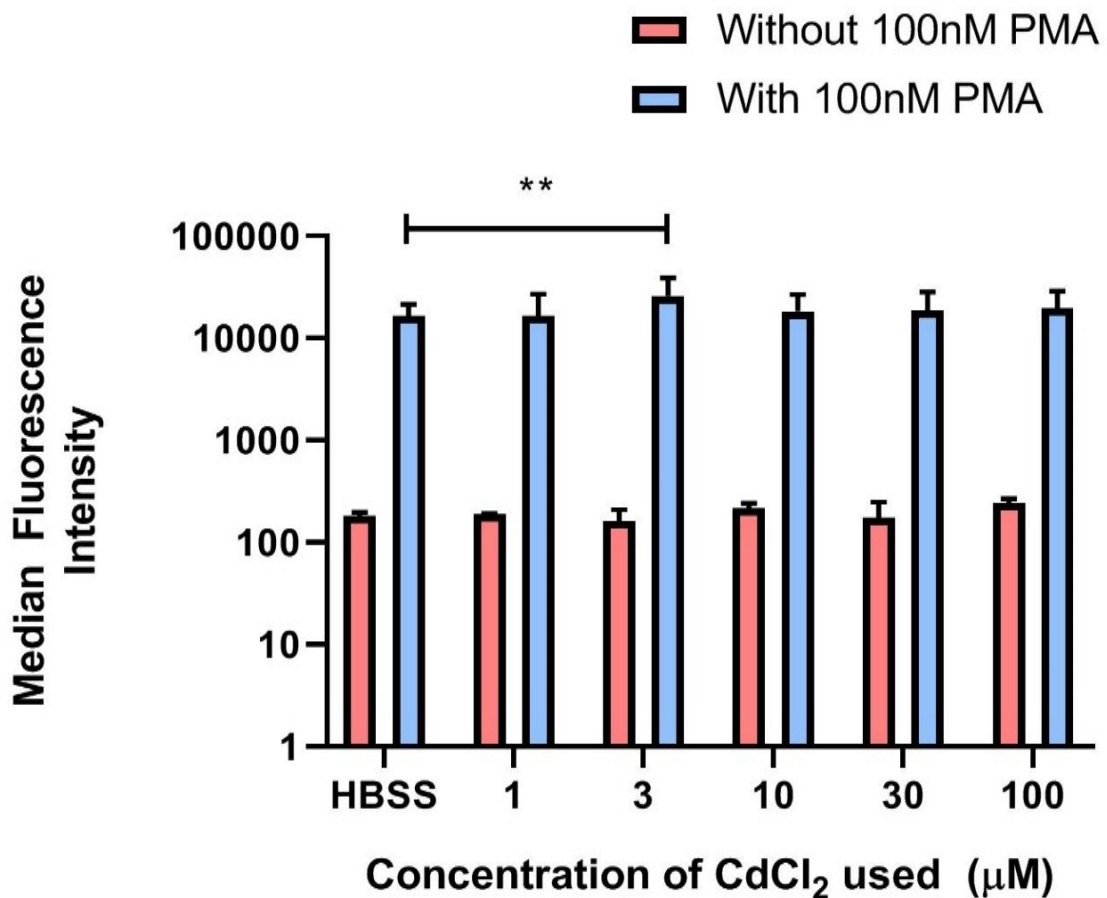


Figure 3-6 The respiratory burst induced by PMA is enhanced by 3μM of cadmium chloride.

Isolated primary neutrophils were incubated with or without 100nM PMA alongside cadmium chloride in the concentrations shown. Production of reactive oxygen species was detected through incubation of neutrophils with 1μM DHR123 alongside treatments. After 30 minutes, green fluorescence was measured. A significant increase in fluorescence was found in neutrophils incubated with 3μM of CdCl₂ and PMA. Statistical significance was tested by within sample two-way ANOVA with Dunnett's multiple comparisons post-test. Data are shown as the mean MFI ± SD over two independent experiments.

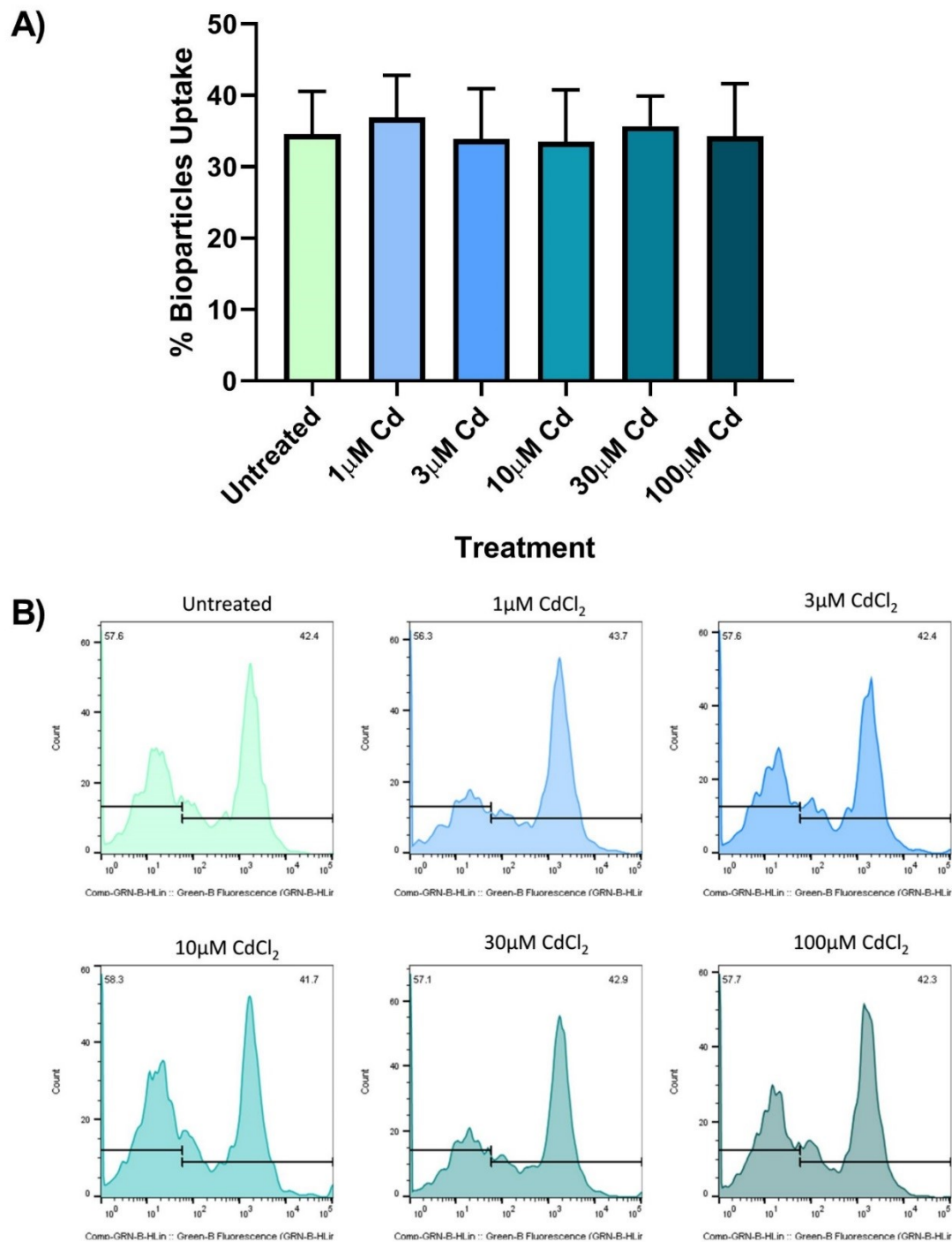


Figure 3-7 Cadmium does alter the rate of uptake of fluorescent bioparticles.

A) Phagocytosis was measured through use of fluorescent labelled *E. Coli* bioparticles. Opsonised bioparticles were added to tubes containing neutrophils at concentration of 1µg/ml along with the treatments indicated above. After 2

hours, fluorescence in each cell was measured to assess phagocytosis. Cells were determined to be particle negative based on gating of 100% of cells that were not incubated with bioparticles and subsequently applied to all samples. Cadmium did not affect the uptake of fluorescently labelled particles. Statistical significance was tested using a Friedman test with Dunn post-test. Data is presented as median MFI \pm IQR over five independent experiments. B) A representative flow histogram of the phagocytosis experiment. Treatment conditions are displayed above each histogram.

In terms of quantity of particles ingested, cadmium also did not influence the median fluorescence intensity in neutrophil populations tested (**Figure 3.8**). Overall, this would suggest that phagocytic capacity of neutrophils is not influenced by treatment of cadmium chloride.

3.4 Neutrophil cytosolic calcium levels increase upon treatment with cadmium.

With conventional neutrophil functions being unaffected by cadmium treatment, along with cadmium demonstrating no cytotoxic effects, it was then investigated whether cadmium could affect certain secondary messenger systems.

Intracellular signalling is reliant on many cascades, though one common molecule that is involved in multiple steps of neutrophil biology is calcium.

Measurement of cytosolic levels of calcium in neutrophils was achieved through usage of the fluorometric dye Fluo-4-AM. Cells were loaded with the dye for 1 hour and then treated with experimental doses of cadmium, or interleukin-8 at a final concentration of 3ng/ml to act as a positive control. Flow cytometry was used to measure the changes in calcium level in each population. Baseline cytosolic calcium level was first measured in the neutrophil population, before spiking in the experimental treatment to monitor any changes in cytosolic calcium levels, then finally adding ionomycin to measure maximum calcium levels inside the cell.

Cadmium induced increases in cytosolic calcium levels beyond those recorded by 3ng/ml of interleukin 8 (**Figure 3.9**).

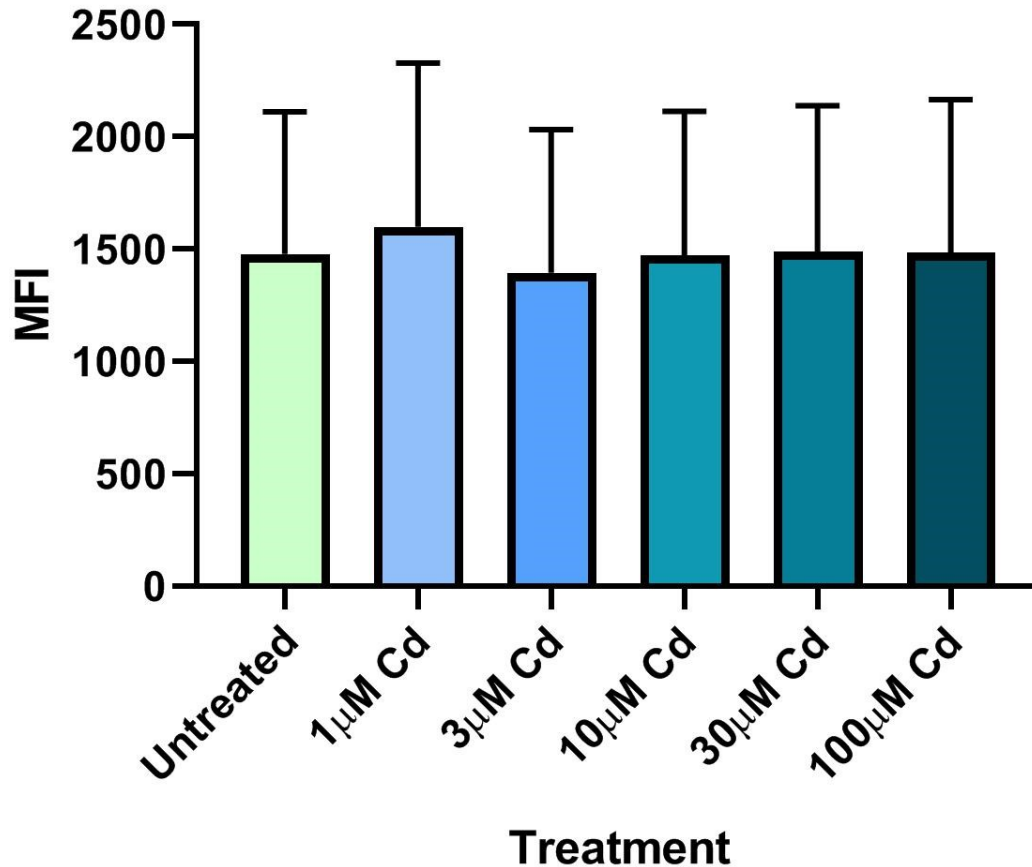
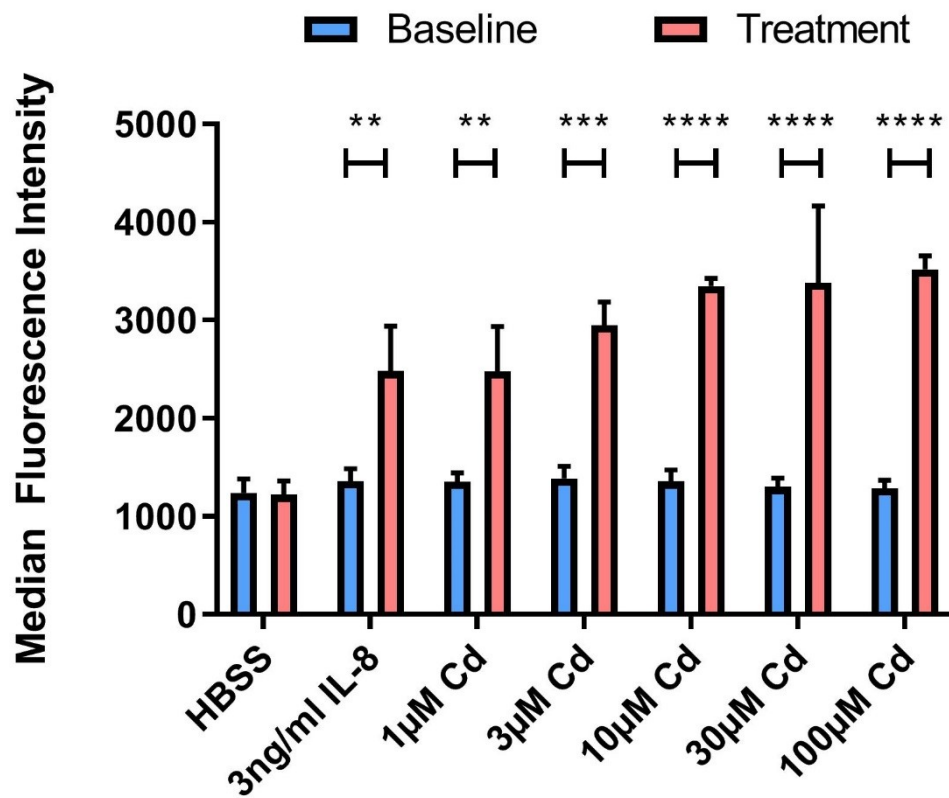


Figure 3-8 A low dose of cadmium does not increase the quantity of bioparticles ingested by neutrophils.

Phagocytosis was measured through use of fluorescent labelled E. Coli bioparticles. Opsonised bioparticles were added to tubes containing neutrophils at concentration of 1 μ g/ml at the same time as cadmium. After 2 hours, fluorescence in each cell sample was measured to assess phagocytosis. Of the cells classified as being positive for bioparticle engulfment, median fluorescence intensity was measured in each population of cells. Cadmium treatment of cells caused no significant increases in bioparticle uptake as assessed by changes in MFI. Statistical significance was tested by Friedman test with Dunn's post-test. Data is presented as the average MFI \pm IQR over five independent experiments.

A)



B)

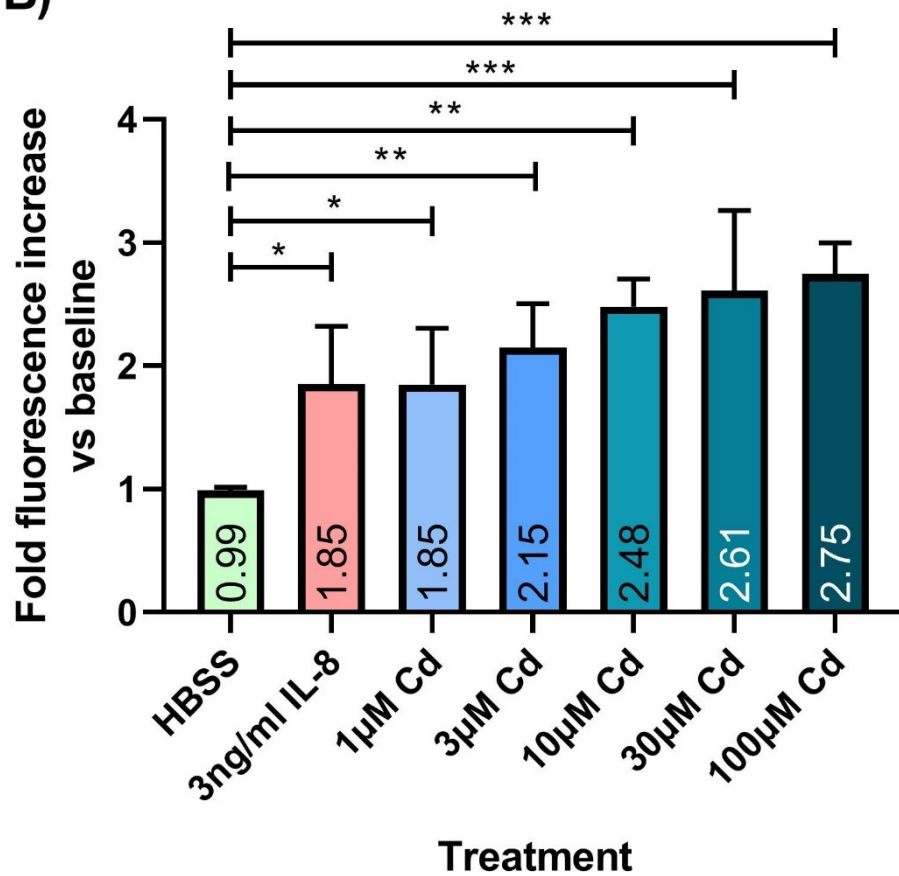


Figure 3-9 Cadmium increases cytosolic calcium levels beyond those induced by low levels of interleukin-8.

Neutrophils were loaded with the fluo-4-AM dye and resting calcium level was recorded indirectly by flow cytometry. After baseline measurement, treatments were added to each sample and the measurements were taken again. A)

Comparison between cytosolic calcium levels before and after treatment with experimental compounds. Cadmium-treatment induced statistically significant increases in cytosolic calcium levels as measured by flow cytometry with some cadmium test concentrations, surpassing the calcium levels induced by IL-8.

Statistical significance was tested by a Within Sample two-way ANOVA with Sidak's multiple comparisons post-test. Data are presented as the average MFI recorded \pm SD over three independent experiments. ** = $p < 0.005$, *** =

$p < 0.001$, **** = $p < 0.0001$ B) The fold increase in cytosolic calcium levels before and after treatments. Cadmium induced a statistically significant, non-dose response increase in cytosolic calcium levels. Statistical significance was tested by a Within Sample one-way ANOVA with Holm-Sidak's multiple comparisons post-test. Data is presented as the average MFI recorded \pm SD over three independent experiments. * = $p < 0.05$ ** = $p < 0.005$, *** = $p < 0.001$.

Every concentration tested (1, 3, 10, 30, 100 μ M) of cadmium chloride induced statistically significant increases in calcium levels ($p=0.0048$, $p=0.0047$, $p=0.0002$, $p<0.0001$, $p<0.0001$ and $p<0.0001$, respectively). A statistically significant positive correlation in calcium levels was observed with increasing cadmium concentrations (spearman's rank correlation coefficient ($r=1.000$, $p=0.0028$)).

3.5 Neutrophil extracellular traps

Neutrophil extracellular traps have been an important focus of neutrophil biology since their discovery (108), with excessive levels of NETosis playing a central role in several diseases throughout the body. In the context of RA, cellular processes which induce higher levels of MPO and PAD during inflammation are believed to play a dual role in both NET induction and autoantigen generation (155). Having examined the effect of cadmium exposure on several common neutrophil functions, attention was focussed on determining if cadmium exposure play a role in altering NETosis. NETosis is a complex process, consequently a number of techniques have been established to measure this process, with varying degrees of success. In this study several NETosis methodologies were established and evaluated with respect to cadmium exposure on neutrophils.

3.5.1 Initial experiments regarding cadmium and NETosis

Initially, overnight neutrophil-cadmium experiments were conducted to discern any neutrophil morphological changes, or evidence of cell death via Diffquik™ staining.

Neutrophils were isolated and suspended into reaction tubes at volumes of 1×10^5 cells and incubated with 1, 2.5, 5, 10 and $50 \mu\text{M}$ of CdCl_2 in RPMI1640 without serum. After incubation, cells were cytopun and Diffquik stained; neutrophils appeared to form protrusions that resemble features of strands of DNA, akin to NETosis (**Figure 3.10**). NETosis by definition is composed of nuclear material released into the extracellular space. To examine if these extracellular strands detected by this method of staining were truly NETs, the experiment was repeated employing an immunofluorescent method.

In brief, 1×10^6 neutrophils were incubated for 16h at 37°C with various concentrations of cadmium chloride and a fraction of cell suspension was spun onto a microscope slide via cytopspin centrifuge at slow speed (200 RCF). Slides were stained for nuclear material with DAPI and citrullinated protein. DAPI staining confirmed that the projections were in fact nuclear material, indicative of NETosis (**Figure 3.11**). Even more interesting was the detection of citrullinated protein in an extracellular space, providing the first suggestion that cellular exposure to cadmium may result in post translational modification of neutrophil proteins (bottom left image in **Figure 3.11**, stained in green).

3.5.2 Immunofluorescent staining of NETs

To confirm the initial findings of cadmium induced NETosis, a further immunofluorescent method was established using HL60-derived neutrophil-like cells.

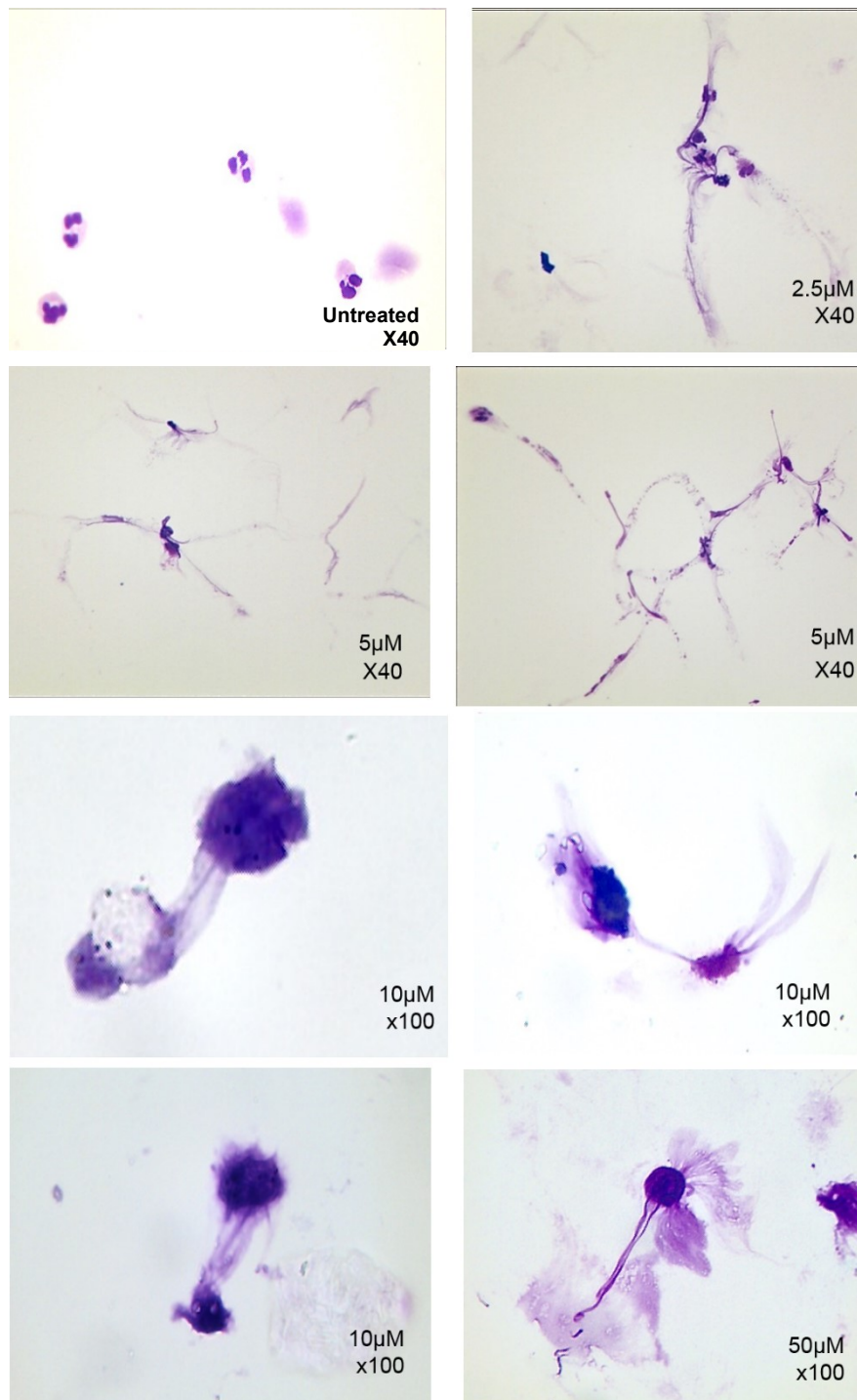


Figure 3-10 Neutrophils produce NET-like protrusions upon treatment of cadmium chloride.

Diffquik staining of treated neutrophil isolates treated with cadmium was performed after a 16 hour incubation. Concentration of cadmium is shown in the bottom right corner. Neutrophils can be seen releasing strands of material into the extracellular space, forming a network of protrusions.

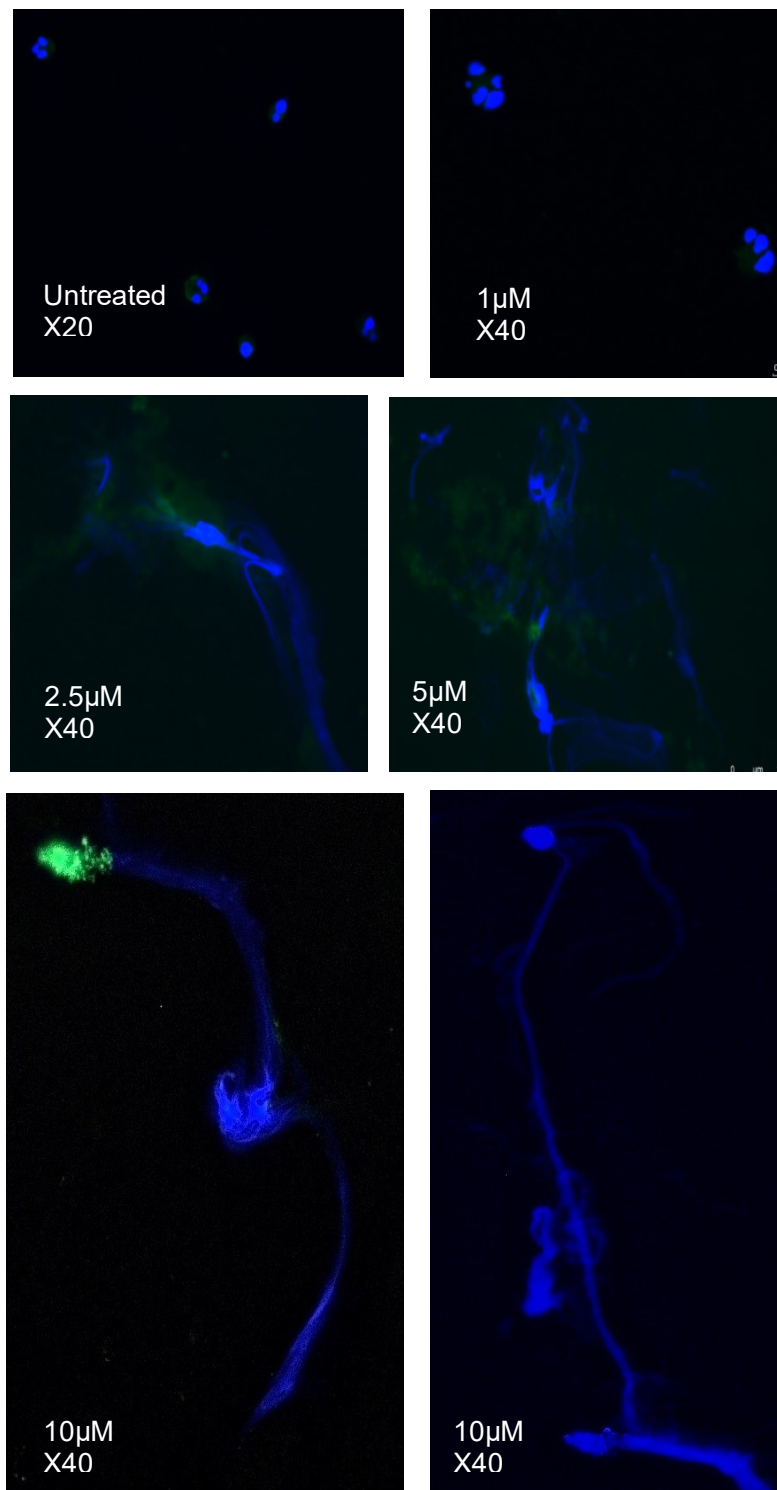


Figure 3-11 Initial immunofluorescent staining of neutrophils undergoing NETosis in response to cadmium.

Neutrophils were probed for citrullinated protein (green) and stained for nuclear material with DAPI (blue). NETs can be observed from as low as 2.5 μ M of CdCl₂. Citrullinated protein can be observed in a cluster of extracellular space at 10 μ M. Concentration of cadmium and magnification for each image can be seen at the bottom-left of each image. Green staining was not universally present in each

HL60 cells were treated with 1-ATRA for 3 days and then incubated with or without 100nM PMA on 8-well chamber slides. After 3 hours, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% v/v Triton-X100. Cells were stained for myeloperoxidase (green) and citrullinated histone-h3 (red), two components found in NETs, in addition to DAPI staining (blue) to confirm the presence of DNA.

Figure 3.12 demonstrates an example of using immunofluorescence to examine NET production. In theory, DNA and MPO should be visible along with citrullinated histone H3. Through immunofluorescence, a triple stained fibre should be connected to the nucleus of the cells. These experiments found no evidence for a co-localisation of all three proteins, suggesting no classical sign of NET production taking place. Links between cells were observed in the form of citrullinated histone H3, though no co-localisation with MPO or DNA was observed, posing the question that without DNA or myeloperoxidase, what exactly is connecting the cells observed in **Figure 3.12**.

Unfortunately, this line of research yielded no clear evidence of NETosis. Although co-localization of citrullinated histone H3 and DAPI was seen in places, the co-localization of the above two proteins and MPO suggested that these may not have been NETs. The lack of consistent sign of NETs in experiments using PMA posed a challenge in being able to determine if cadmium was capable of inducing NETosis, and so an alternative approach was used.

F

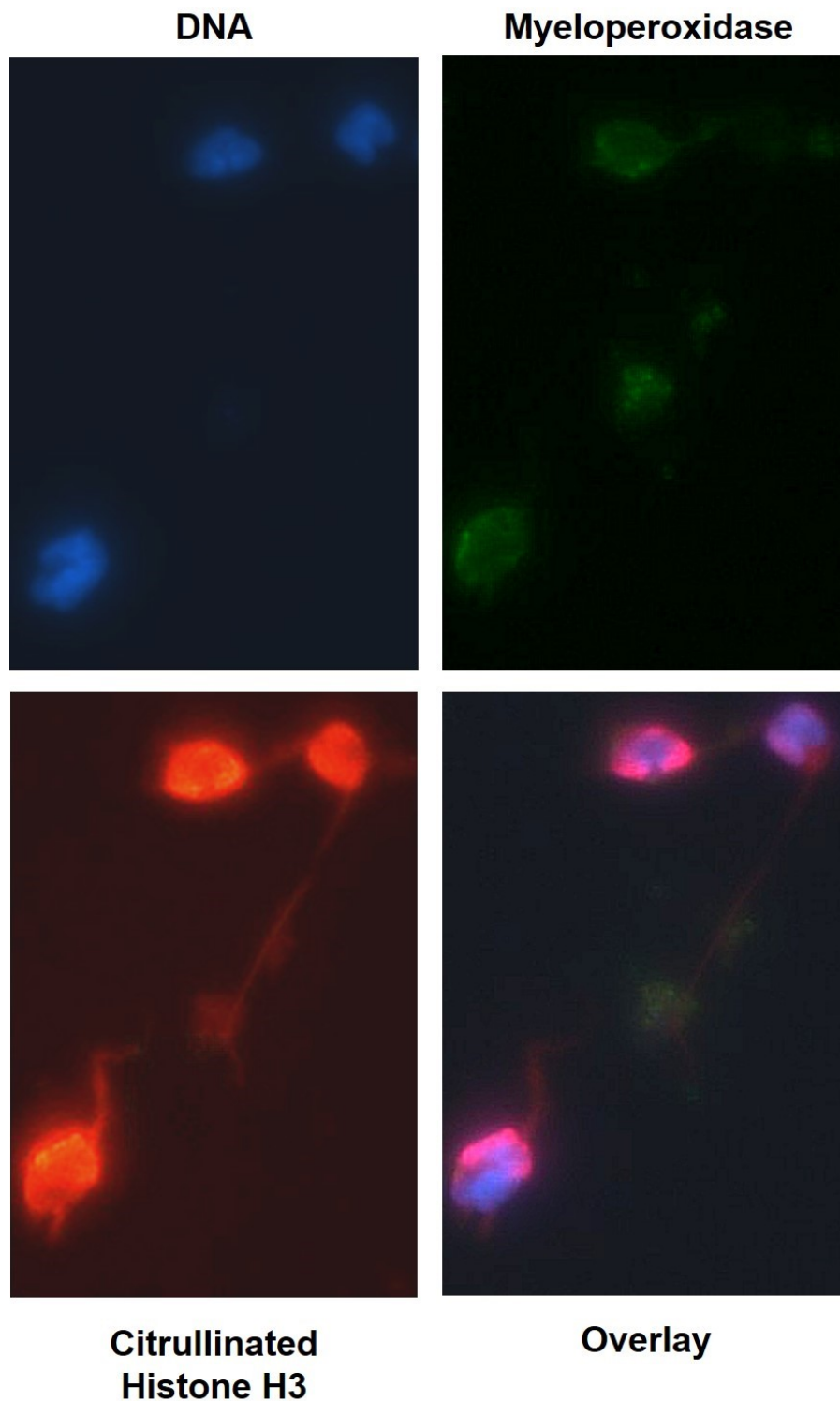


Figure 3-12 Citrullinated histone H3 is present in projections from HL60 neutrophils, but not co-localised with DNA or myeloperoxidase.

HL60 derived neutrophils were stimulated with PMA and immunofluorescent staining of citrullinated histone H3 (red), myeloperoxidase (green) and DNA (blue). Images were taken at 40x magnification.

3.5.3 Live cell imaging of neutrophils induces a form of cell death separate to apoptosis

In order to abrogate the inconsistency posed by immunofluorescence imaging of individual cells at a given time point, live cell imaging was chosen as a means to determine if NETosis was occurring in a population of cells over time.

Live cell imaging on the JuLi stage cell recording system was used to establish a more objective and quantitative approach for monitoring NET induction.

Before using primary neutrophils, HL60 neutrophils were employed to establish the method for routine use.

HL60 neutrophils were loaded with Dil (Invitrogen, UK) to visualise cells, and then seeded onto 96-well plates in serum-free media in order to adhere to the bottom of the microplate. During this time focal planes were set before adding the agonist and fluorescent probes. 100nM PMA alongside both Hoescht stain for detection of cellular DNA, as well as SYTOX green, a cell impermeable DNA dye used widely to monitor NETosis, was used to detect extracellular DNA.

The results from live cell imaging demonstrated an interesting phenomenon separate to apoptosis that differed from conventional NETosis. Cells treated with 100nM PMA rapidly took up Sytox dye inside the cell, before forming a circular ring around the cell. In **Figure 3.13** evidence of this process can be seen, with cells indicated by green circles rapidly forming a ring around the cells kept closely associated with the cell membrane.

The argument for this being NETosis lies upon the postulation that in live-cell imaging, no source of shear flow is exerted on the cells, therefore unless nuclear DNA is forcibly ejected from the cell, it would remain associated with the neutrophil or bound to the plastic immediately surrounding the cell.

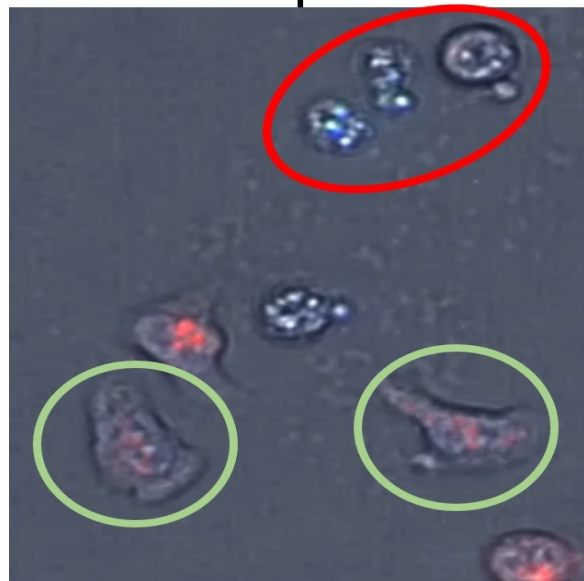
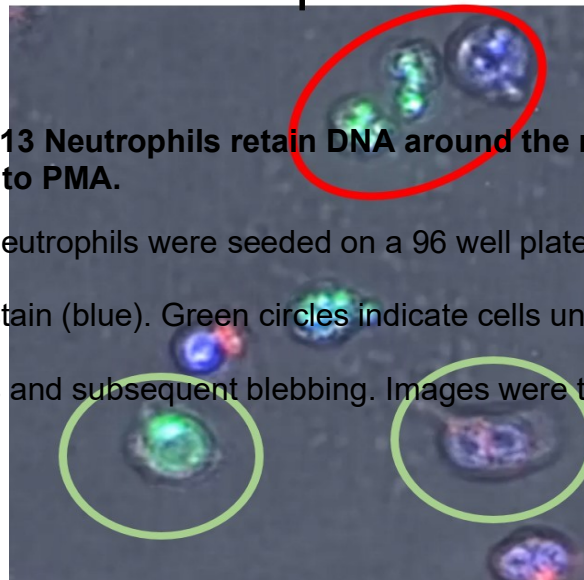


Figure 3-13 Neutrophils retain DNA around the membrane of the cell in a death process exposed to PMA.

Primary neutrophils were seeded on a 96 well plate and exposed to 100nM PMA in addition to DAPI (blue) and propidium iodide (red). Green circles indicate cells undergoing the process of “haloing”, whilst red circles indicate cells undergoing apoptosis and subsequent blebbing. Images were taken 5 minutes apart at 20x magnification.

Figure 3.13 also demonstrates that this is different to apoptosis, as indicated by the red rings. Membrane blebbing can be observed in this population of cells, and the dissimilarity of the two ringed populations highlights a greater chance of NETosis than apoptosis or necrosis.

To interrogate this further, a more biochemical approach was optimised to assay NETosis, using a SYTOX green spectrofluorometric assay. To provide more consistently reproducible data that could directly quantify SYTOX green fluorescence, a plate-based high-throughput bioassay was used. First demonstrated by Gupta and colleagues, this assay allows an easy, reliable method to quantify NETosis (120). The principle behind this assay is to directly measure the green fluorescence emitted by SYTOX green upon extracellular DNA binding using a spectrofluorometer, and comparing cadmium treated samples against untreated and PMA-treated populations of neutrophils. This then allows the extra validation of generating data points that can undergo statistical analysis to determine if cadmium does truly induce NETosis.

3.5.3.1 PMA reliably induces NETosis in a spectrofluorometric SYTOX green assay

A concentration-response of HL60-derived neutrophils treated with PMA (10-100nM) was performed to find as low a concentration as possible that was capable of inducing NETosis as PMA is not a physiological source of PKC activation (**Figure 3.14**).

HL60-derived neutrophils were seeded in 96 well plates and allowed to adhere to the plate for an hour. After this, varying concentrations of PMA were added to

each well and incubated for 3 hours. After incubation, SYTOX green was added to detect extracellular DNA (exDNA) and therefore quantify NETosis.

Levels of extracellular DNA detected by SYTOX green upon PMA stimulation were within similar quantities between the concentration windows of 50-100nM of PMA. At lower concentrations, SYTOX green still detected increases in exDNA, though at diminished levels before 50nM. With this in mind, 50nM was chosen for future experiments as the optimal concentration to use for SYTOX assays.

3.5.3.2 Cadmium does not induce NETosis as determined by the SYTOX green assay

Once the positive control concentration was established, this system was tested against various stimuli to determine if cadmium does truly induce NETosis. In addition to cadmium, additional pathological agonists based on the literature were used. Bronchiectasis patients with chronic lung infections often are infected with *P. aeruginosa*. It was therefore warranted to test if LPS from this microbe was capable of inducing NETosis in a similar manner to other species reported in the literature, such as *E. coli*. In addition, a further chemical inducer of NETosis, ionomycin, was included as a positive control. None of the concentrations of cadmium tested significantly changed any exDNA levels detected by SYTOX green (**Figure 3.15a**). In addition to this, none of the concentrations of LPS tested changed exDNA levels compared to control. Ionomycin significantly increased exDNA levels compared to control ($p=0.0001$).

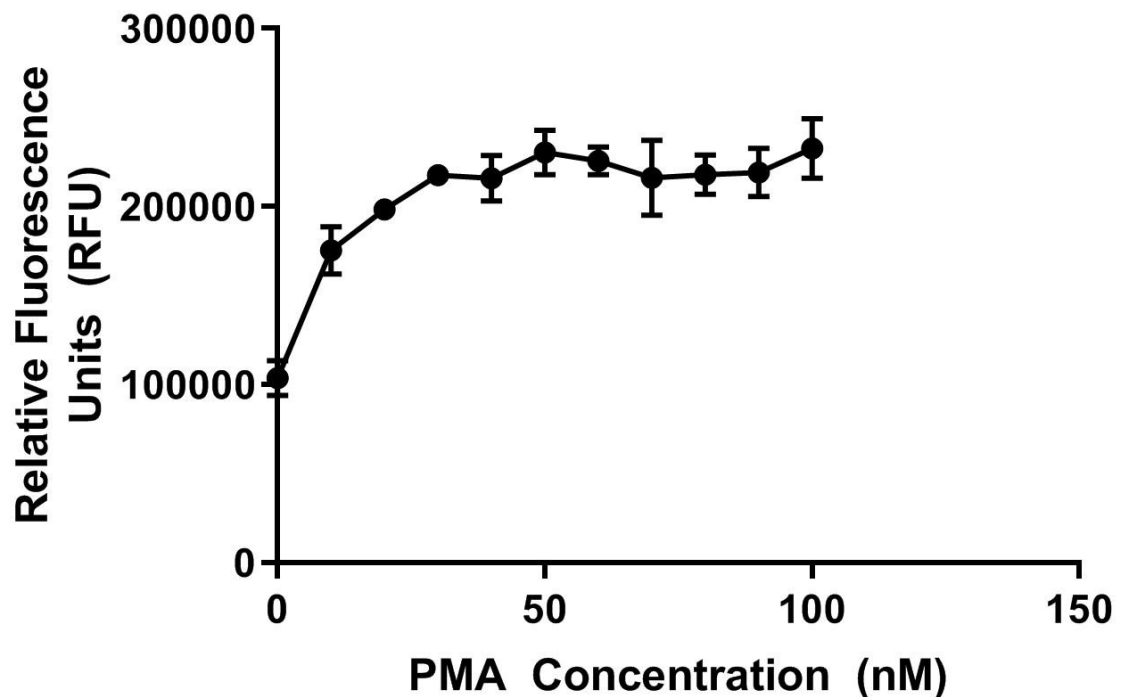


Figure 3-14 Consistent levels of NETosis can be induced by PMA from 50nM concentrations.

HL60 derived neutrophils were treated with 10-100nM concentrations of PMA and incubated for 3 hours to allow NETosis to occur. SYTOX green was added to the plate post-incubation and green fluorescence levels were recorded. 50nM induced similar levels of NETosis to higher concentrations used. Data are shown as relative fluorescence units of green fluorescence over three independent experiments. Data values are presented as mean RFU recorded \pm SD over three independent experiments.

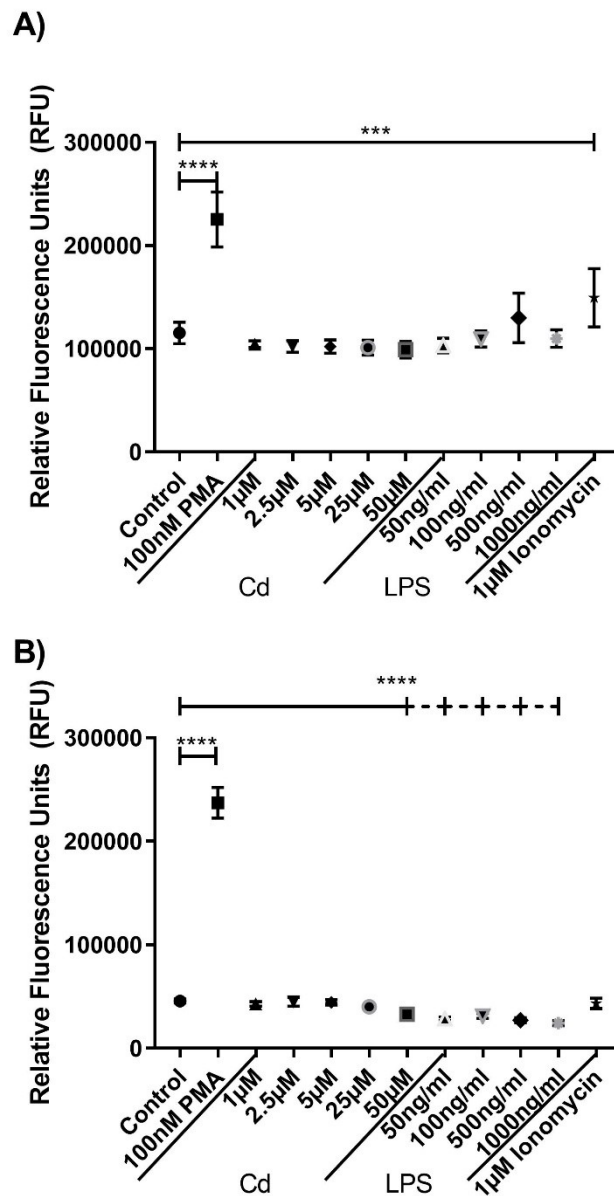


Figure 3-15 Neither cadmium, nor LPS results in a significant increase in extracellular DNA levels.

Isolated primary neutrophils were treated with various doses of cadmium chloride and lipopolysaccharide, alongside ionomycin. Cells were incubated for: A) 3 hours or B) 24 hours before adding SYTOX green to measure extracellular DNA levels. Data are presented as relative fluorescence units \pm SD for three independent experiments. Statistical significance was tested by one-way ANOVA with Holm-Sidak's post-test. *** = $p < 0.001$ **** = $p < 0.0001$. Dotted lines in 3.14b indicate individual comparisons to the control samples.

These data would suggest that neither cadmium nor LPS induce NETosis in a 3h time window. In order to further evaluate if the neutrophil response was delayed, these experiments were repeated with a 24h incubation, before measuring exDNA.

Upon repeating this experiment with a 24h incubation window, all concentrations of LPS tested (50, 100, 500 and 1000ng/ml) significantly lowered the levels of extracellular DNA in neutrophils ($p < 0.0001$ for all concentrations – **Figure 3.15b**). Cadmium also exhibited this effect at the 50 μ M concentration, with significantly lower exDNA being detected compared to control samples ($p < 0.0001$). Lower concentrations of cadmium caused no significant changes in exDNA levels (**Figure 3.15b**).

With the effect of direct cadmium exposure not eliciting any response in terms of direct NET induction, any effect of cadmium on PMA-induced NETosis was examined. Firstly, HL60-derived neutrophils were pre-incubated with cadmium chloride for 16 hours in low concentrations (1, 2.5, 5, 10 and 15 μ M). Cells were then treated with 100nM PMA for 3 hours before quantifying DNA with SYTOX green.

Cadmium pre-incubation exhibited no modulatory effect on NET induction in PMA-treated neutrophils (**Figure 3.16**). Statistical analysis showed significant differences between the Cd-untreated and Cd-PMA treated cohorts. No significance was observed between each cadmium treatment group.

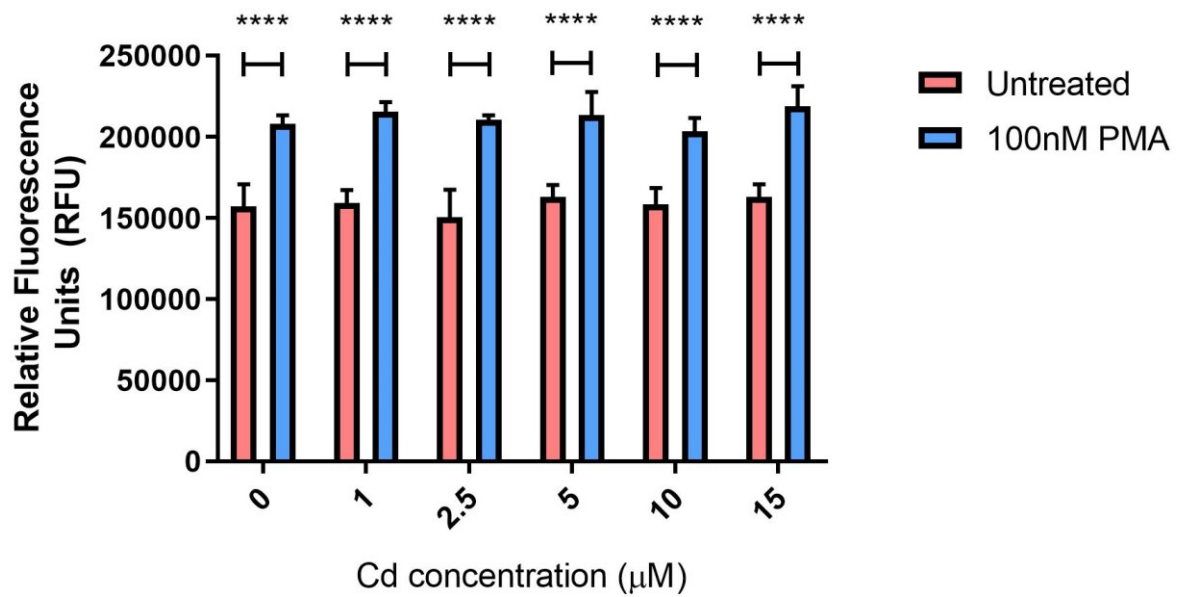


Figure 3-16 Overnight pretreatment of cadmium does not enhance NETosis in PMA-treated neutrophils.

HL60-derived neutrophils were seeded and incubated for 16 hours with low doses of cadmium chloride. After this time, cells were exposed to 100nM PMA to induce NETosis. After 3 hours, SYTOX green was added to the plate and green fluorescence was measured. Statistical significance was tested by a within sample two-way ANOVA with Sidak's multiple comparisons tests comparing RFU's between untreated and PMA treated populations, in addition to testing for significance between each individual treatment in each group. No statistical significance was observed between any of the PMA-treated neutrophils. Data are shown as mean RFU \pm SD over three independent experiments. **** = $p < 0.0001$.

As an enhanced level of ROS production in cadmium and PMA treated neutrophils had previously been observed, the effect of concurrent stimulation with cadmium and PMA on NETosis induction was also considered. HL60-derived neutrophils were co-incubated with the same treatment doses as observed in **Figure 3.16**, for 3 hours. Intensity of NETosis was once again conducted by measurement SYTOX green fluorescence.

Of all the treatment dose of cadmium used, 15 μ M Cd co-incubated with PMA caused a statistically significant increase in the quantity of DNA release through PMA-induced NETosis compared to “no cadmium” treatment ($p < 0.001$) (**Figure 3.17**). No other treatment of cadmium demonstrated statistically significant changes in DNA levels compared to the untreated group.

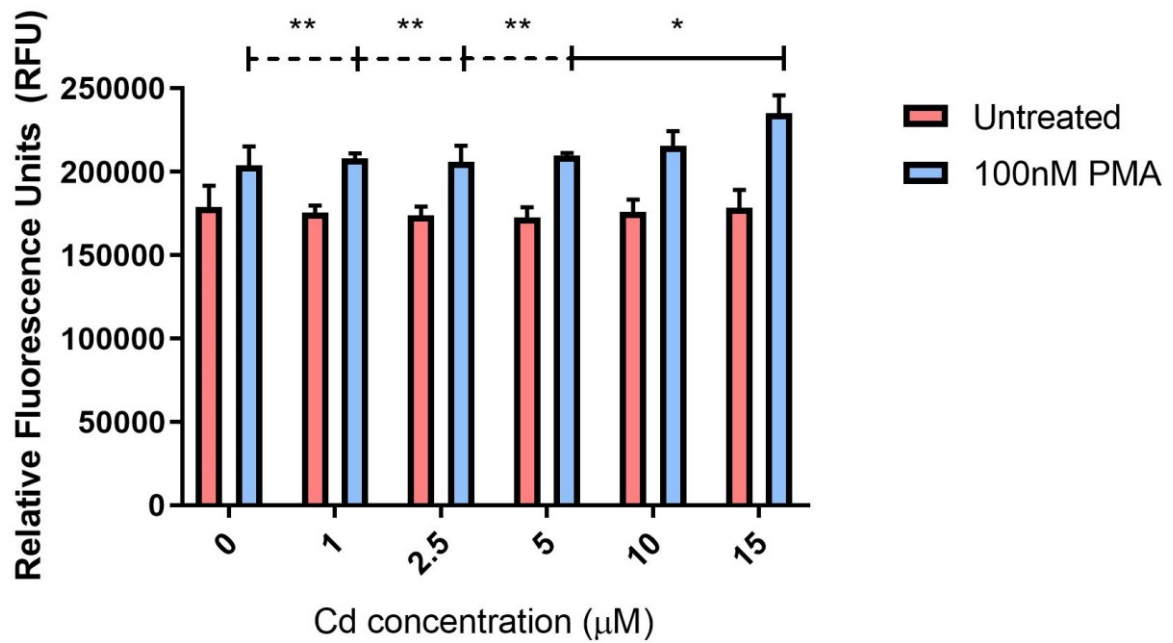


Figure 3-17 Co-treatment of cadmium enhances levels of NETosis at higher concentrations.

HL60-derived neutrophils were seeded and incubated for 3 hours with cadmium chloride and 100nM PMA. After 3 hours, SYTOX green was added to the plate and green fluorescence was measured. 15μM of CdCl₂ induced a significant increase in extracellular DNA compared to any other treatment used. Statistical significance was tested by a within sample two-way ANOVA with Sidak's multiple comparisons. Dotted lines represent comparisons between 15μM CdCl₂ treatment and the treatment dose in question. Data are shown as mean RFU ± SD over three independent experiments. * = p<0.05 ** = p<0.01

3.6 Discussion

Previous studies have demonstrated effects of cadmium on neutrophil function (314, 323). These findings were expanded, using a wider range of cadmium concentrations in these experiments, focusing on phagocytosis, calcium flux and NETosis. Under the test conditions described in this thesis, cadmium did not appear to have an adverse effect on any of neutrophil functions examined, although cadmium did induce changes in cytosolic calcium levels. Contrary to initial findings, cadmium did not induce NETosis when tested via the plate-based SYTOX green assay. Of interest was the lack of NETosis induced by LPS, which has historically been shown as a potent inducer of this process. Recently, cadmium has been shown to influence lung injury by enhancing neutrophil extracellular traps via a ERK1/2 and p38 MAPK signalling pathway (324). However this latter study was performed in mice and might not translate to human cells. In the current work human neutrophils are seemingly resistant to cadmium treatment.

3.6.1 Effect of cadmium on cell viability

Cadmium is a known cytotoxic agent for a variety of cells in the body (24-29). In light of this, the lack of cytotoxic response in neutrophils is puzzling, given the short-lived nature of the cell, and propensity to die when exposed to toxic stimuli during bouts of infection, for example (325). Neutrophils have been previously incubated with cadmium chloride for 48 hours at concentrations ranging between 0-200 μ M in concentration (326). Apoptosis was found to increase from 5% in untreated cells, to 40% in neutrophils exposed to 300 μ M, in a dose-responsive manner (326). The explanation for differences observed may be due to the conditions through which the neutrophils were treated. In the study by de

la Fuente *et al*, isolated mononuclear cells were resuspended in RPMI-1640, supplemented with foetal bovine serum, in addition to penicillin and streptomycin. This work resuspended neutrophils in HBSS without any other forms of supplementation, apart from 10% autologous serum, in order to prevent any other influence to cell longevity than the treatment compound in question. Even in the untreated group, cells were readily undergoing apoptosis within 24 hours, as is accepted to be the standard lifespan of neutrophils in the body (327).

It may well be that the concentrations of cadmium that were used in these experiments were simply too low to exhibit cytotoxicity in neutrophils.

Macrophages are resistant to apoptosis when exposed to cadmium, requiring a concentration of 200 μ M in order to undergo apoptosis. This suggests that the phenomenon of cadmium resistant cytotoxicity may not be restricted to neutrophils alone (300).

A human is thought to possess approximately 26ml of lung lining fluid (328).

From this, it may be postulated that an inhaled dose of 2.2 μ g Cd from a cigarette would result in the lung fluid containing up to 1 μ M of cadmium (although localised concentrations could be higher), which then poses the question of how relevant the tolerance limits are for immune cells when such levels of exposure are unlikely to be encountered *in vivo*? The levels of cadmium in the lungs may be further estimated through measurements of cadmium in BAL fluid of smokers, which found a concentration 0.8 μ g/L, which translates to 0.185nM resting concentration, further lending credence to the 10% lung retention of cadmium estimated by Mannino *et al*. (6, 7, 65).

3.6.2 The effect of cadmium on neutrophil function

It has been reported that cadmium diminishes the response of the respiratory burst in neutrophils and macrophages when challenged by bacterial stimuli (329). This has been theorized by Loose *et al* to be due to an inhibitory effect of cadmium on myosin ATPase (329). This was subsequently disproved by Nimura *et al*, who found that cadmium actually plays a stimulatory role on myosin ATPases, with cadmium ions mimicking the role of Ca^{2+} ions and subsequently enhances enzyme activity (330). In plants, cadmium increases levels of H_2O_2 , through interaction with NADPH oxidase (331-334). In macrophages, cadmium increases production of superoxide and nitric oxide over a 48 hour period from 400nM exposure (335). This may be due to activation of oxidative stress inside the cell, rather than the immunological function of the respiratory burst, however. Freitas *et al*. found that the respiratory burst in neutrophils was induced by 500 and 1000 μM of CdCl_2 in a luminol chemiluminescence assay (336). Furthermore, these same concentrations also enhanced ROS production in PMA stimulated neutrophils. Freitas *et al*. treated neutrophils with PMA and cadmium in a similar experimental style as performed in this thesis, though only uses a blank as a control for the experiment, not a negative treatment control. However, Freitas *et al*. used a luminol chemiluminescence assay to measure ROS and reported production in neutrophils from 0.5-1mM treatments. However, luminol directly interacts with various heavy metal ions, including copper and cadmium, which may be giving false-positive reads in assays due to direct chemical reaction, as opposed to inducing production of ROS which then reacts with the detection agent (337, 338).

Contrastingly, Zhong *et al.* reported no production of ROS upon cadmium stimulation, providing conflicting reports as to whether or not cadmium induces the respiratory burst in neutrophils (339). These data agree in part with the latter study: It has been shown that cadmium by itself does not induce the respiratory burst in neutrophils through usage of the highly accurate DHR123 dye (322). However, this work has also shown that cadmium enhances production of ROS in neutrophils stimulated by PMA. This resembles similar findings by Ramirez *et al.*, who noted increases in nitric oxide production in macrophages incubated with PMA and cadmium, as opposed to PMA alone. Taken together, this may suggest that cadmium by itself does not affect the respiratory burst in neutrophils, though may prime them for an even stronger response once stimulated by other means (340).

The process of phagocytosis appears to be modulated somewhat by exposure to cadmium. Greenspan *et al.* noted a diminished phagocytic response in macrophages exposed to 100 and 200 μM of CdCl_2 , remaining relatively unmodified when macrophages were exposed to 20 and 50 μM concentrations (341). These results somewhat reflect these findings, as phagocytosis was unaffected by any of the concentrations of cadmium chloride that were tested. These differences may simply reflect the different cell types utilised, i.e. primary human neutrophils versus alveolar macrophages from rats. Levy *et al.* reported no influence of cadmium on the ability for macrophages to bind to immunoglobulins through Fc receptors, or complement through complement receptors, though did affect the process of endocytosis, with cells displaying a delayed ability to clear ingested particles (323).

Although not ideal, hemocytes (from molluscs) are comparable to human neutrophils, so allow us to somewhat draw parallels to mammalian cell

functioning (342). Research on the effect of cadmium on hemocyte phagocytosis found reduced phagocytic capacity of cells, though the authors concluded this may be as a result of reduced cell viability (343). 1mM of CdCl₂ inhibits 20-70% of phagocytosis in a variety of hemocytes isolated from various bivalve species, though this was unaffected in concentrations lower than this (344).

A common theme can be noticed throughout these positive results in the literature. Interference of phagocyte functions is perturbed by cadmium only at supra-physiological levels of 100µM or above. This far exceeds the physiological levels that would likely be encountered by cells in the body, and is the main reason why it was decided to keep the experimental concentrations in this thesis between 1-100µM.

In all the assays used, cadmium did not directly stimulate any neutrophil functions. The only response observed was during the respiratory burst, in which neutrophils stimulated with PMA appear to produce a greater amount of ROS in the presence of low concentrations of cadmium. Phagocytic capacity was not affected by cadmium treatment, nor did it affect the quantity of particles internalised by phagocytosis.

Calcium levels inside cells have long been known to increase upon cellular exposure to cadmium (345). Of all the heavy metals (cadmium, cobalt, nickel, iron and manganese,) cadmium evokes the strongest release in a variety of cells, typically increasing calcium levels 10-fold (345, 346). In murine neutrophils, Mei *et al.* reported a diminished calcium mobilisation response in cadmium-pretreated neutrophils upon stimulation with fMLP (347). The authors also reported reduced levels of phosphorylation of p38 and ERK1/2, indicating

that cadmium may interfere with cell signalling pathways in these cells (347).

The authors concluded that this may interfere with chemotactic response to bacterial peptides.

This data is in line with the literature, in that it has shown that in human neutrophils, cadmium induces an increase in cytosolic calcium levels. This increase may affect the second messenger systems inside the cell, which may result in aberrant responses to external stimuli. Protein kinase C activity is enhanced through cadmium displacing the Zinc ion on the enzyme, resulting in a greater response when stimulated by PMA in fibroblasts (348). This may account for the greater respiratory burst when cadmium was co-incubated with PMA.

The lack of reactive oxygen species production from cadmium alone in these experiments however, would indicate that cadmium may not be directly stimulatory in neutrophils. The effect may be more of a priming nature, in which neutrophil activity is enhanced by cadmium, to an exaggerated degree. This effect could partly explain cadmium-induced inflammatory processes.

3.6.3 The controversy of NETosis in the scientific literature

NETosis remains an elusive concept in research in terms of how reliable published literature is in reporting NETosis through experimental means. Konig *et al* reported various issues with groups reporting NETs, and how valid were the findings (111). The authors argue that certain NET process that have been reported as NETs may in fact be morphologically similar, yet still different. They also argue that NETosis is NADPH oxidase dependent, any form of NET that does not utilise this pathway is in fact not NETosis.

During the process of discerning if cadmium induces NETosis in neutrophils, several conflicting points were encountered. Firstly, the standard culture conditions used to induce NETosis are contradictory in that culture medium contains certain compounds that theoretically inhibit the detection of neutrophil extracellular traps. Foetal bovine serum, a vital component of tissue culture practice, contains DNases that degrade nuclear material, in addition to antioxidants that may attenuate ROS production required for NET creation (349, 350). Additionally, tissue culture media are frequently supplemented with penicillin/streptomycin. Interestingly, streptomycin inhibits PAD4 in micromolar concentrations (351). Standard media preparation estimates that 1% streptomycin equates to roughly 17 μ M concentration, therefore would strongly inhibit PAD4, resulting in a lack of appropriate activation during pathways that may be citrullination-dependent.

Furthermore, using cytopins as a means of cellular fixation before conducting immunofluorescent staining may lead to false-positive results. This preliminary data found evidence of NETosis upon overnight incubation of cadmium. Upon subsequent repeats, it was evident that the fibres observed through microscopy although appearing to be NETs, were possibly nuclear material that was released outside of the cells during the centrifugation process. Certain studies use this technique to discern NET production, but it may be wise to avoid this approach to ensure accurate depiction of NETs (352-354).

Live cell imaging as reported in the literature also poses several questions pertaining to the reliability of the data. Some studies show results typical of those found via immunofluorescent staining (113, 120). These data demonstrated the formation of ring-like structures that were anchored to the membrane of cells that stained positive for SYTOX green upon stimulation with

PMA. In live cell imaging, cells are focused and observed over a period of time to monitor changes through use of various dyes. In this system, no source of shear flow is present to exert any force on the cells and therefore direct movement of DNA to form the typical strands of DNA observed by immunofluorescent staining. In the study by Gupta *et al*, for example, a probable source of lateral flow is required to tease out the strand-like SYTOX green signal during NETosis (120).

It is acknowledged that visualisation of NETosis can be challenging, such that newer dyes (such as cytotox green) are being manufactured that may improve the reliability of data obtained using live cell imaging (355).

After trying immunofluorescence and live-cell imaging, the most reliable data was obtained through spectrofluometric quantification of extracellular DNA using SYTOX green. This assay provided high throughput, quantifiable data, though presents caveats itself. Firstly, SYTOX green is a non-permeable DNA dye, which in theory has several strengths in quantifying NETosis, as it theoretically only binds to DNA from cells that have undergone the process of NETosis. However, this dye also binds DNA from cells that have undergone apoptosis, as shown from the live cell imaging data. This potentially means that the spectrofluorometric assay could detect both NETosis and apoptosis, though given these results only show a significant increase in DNA levels upon PMA treatment, the question really is if PMA induces apoptosis in neutrophils. Saito and colleagues have reported PMA-induced apoptosis, citing observations of chromatin condensation upon treatment independent of caspase-3 (356). This is not the first time PMA-induced apoptosis has been reported, though both Takei *et al*. and Saito *et al*. state that this was a form of cell death separate to conventional apoptotic pathways (127, 356), and may therefore have been

NETosis, with these studies being published before the ground-breaking work by Brinkmann *et al.* opened the door to this new insight on neutrophil biology (108). It can therefore be assumed that PMA may be inducing negligible amounts of cell death in neutrophils compared to the large quantities of NETosis usually reported.

More interestingly, no levels of NETosis were observed upon stimulating neutrophils with LPS. This has historically been shown to stimulate neutrophils into undergoing NETosis (reviewed in (113)). The reasons for this may be due to lack of second stimulus such as ATP, as LPS is thought to prime neutrophils before subsequent activation by another molecule (357). Nevertheless, Brinkmann and colleagues still observed formation of NETs in response to LPS alone (108).

Another explanation for the lack of NET induction may be due to pH of the media in question. Lipopolysaccharide-induced NETosis is influenced by pH, with higher pH inducing a greater level of NETs, being non-existent at pH 6.6, yet substantially increasing at pH 7.8 (358). As pH was not factored in during the design of these experiments, nor was this novel data available at the time, this may also have impacted on these data.

3.6.4 Conclusion

Based on these data, it can be stated that cadmium has no clear effect on neutrophil function. However, it appears that cadmium may prime, or modulate neutrophil responses through enhancement of protein-kinase C function, which in turn results in greater responses from activation of this pathway when further stimulated by PMA. The increase in cytosolic calcium levels upon cadmium exposure may also influence other secondary messenger systems inside the

cell, though which functions may be affected by this remains unknown. These changes ultimately result in greater levels of ROS production and NETosis, which has consequences in inflammatory events, as this enhanced response may result in host tissue damage. The argument may be made once again that 15 μ M of CdCl₂ is not physiologically relevant, however the use of greater concentrations of cadmium in the cadmium-PMA co-incubation SYTOX green assay may reveal a more exaggerated response than what has already been observed. The lack of apoptotic cell death upon cadmium exposure highlights that those pathways are not initiated. Having examined whether cadmium may contribute to RA pathogenesis through neutrophil dysfunction, it was decided to examine the effect of RA serum on neutrophils, to determine if serum from patients with varying degrees of clinical severity had any direct influence on neutrophil functions *in vitro*.

4 The effect of rheumatoid arthritis patient serum on the neutrophil response

4.1 Introduction

Neutrophils are present at all stages of RA pathology, from both the onset of the disease, to the ongoing pathology once immune tolerance has broken down (245). The objectives of this chapter were to:

- Examine the effect of RA serum on the neutrophil respiratory burst, NETosis and calcium flux
- Ascertain if RA serum from patients with varying disease severity correlates with any dysfunctional responses seen in neutrophils
- Investigate whether elevated levels of individual clinical parameters (anti-CCP, RF, DAS-28) in RA serum contribute to altered functioning of healthy neutrophils

4.2 Cohort serology

Sera from RA patients across the South-West of the UK were gathered based on a variety of patient selection criteria. The intention with this cohort was to discern the influence of cadmium levels (determined through measurement of patient urine samples) on clinical serological scores. The concentrations of two stable isotopes of cadmium (Cd-111 and Cd-112) were independently measured at the SAS Trace Element Laboratory in Guildford, Surrey, by inductively coupled plasma mass spectrometry. The average values in nmol/L were calculated between these two isotope cadmium species and used as a value for clinical correlation analysis. In addition to urine samples, serum was collected from over 200 individuals, with all patient history including smoking status, age and urinary cadmium level (**Table 4.1**). Measurement of urinary

cadmium levels for the population were performed before the project began, though ideally, serum measurement would have been preferable.

	Age	Sex (% Female)	Smoking Status (Ever%)	Rheumatoid Factor (U/ml)	Anti-CCP (U/ml)	DAS-28 Score	Urinary Cadmium Level (nmol/L)
Minimum	36			0	0	0.9800	0.05
Median	69			72.60	193.5	3.070	0.4511
Maximum	87			1000	1000	19.00	7.38
Mean	67.7	41.7	66	154.2	217.2	3.254	0.5887

Table 4-1 A summary of RA patient cohort exposed to varying levels of cadmium.

This table summarises the various clinical history of the patient serum samples collected in 205 RA p

A Spearman's rank correlation coefficient was used to compare urinary cadmium level against RF, Anti-CCP, DAS28 and age. A highly statistically significant correlation was observed between cadmium and RF levels ($P=0.0004$, $r=0.2393$). To confirm the general correlation between RF and CCP typically found in RA patients, a Spearman's rank correlation was performed between these two parameters (359). Rheumatoid factor titres statistically significantly correlated with anti-CCP ($p < 0.0001$, $r = 0.3395$).

To further discern the influence of cadmium on clinical measurements, the cohort was sorted by urinary cadmium level into upper and lower (75% and 25%) quartiles. An analysis was done to compare RF, anti-CCP and DAS28 scores between the upper and lower quartiles and tested for statistically significant differences.

The low-cadmium quartile had levels between 0.054 and 0.296 nmol/L of cadmium, and the high-cadmium quartile ranged between 0.7902 and 7.3 nmol/L. Statistical analysis through spearman's rank correlation coefficient demonstrated that RF titres were significantly higher ($p=0.0017$) in the cadmium-high group compared to the cadmium-low group (**Figure 4.1**). Anti-CCP titres and DAS-28 scores were not significantly different between these two groups ($p=0.4682$ and $p=0.0878$, respectively).

To evaluate any further potential relationships between cadmium and RA, the cohort was also divided into ever and never smokers. Comparisons between RF, CCP, DAS28 and urinary cadmium levels in these two groups were calculated (**Figure 4.2**). Urinary cadmium levels were statistically significantly

higher in the ever smoking group ($p=0.0014$), as expected. Rheumatoid factor was also significantly higher in the ever smoking cohort ($p=0.0351$).

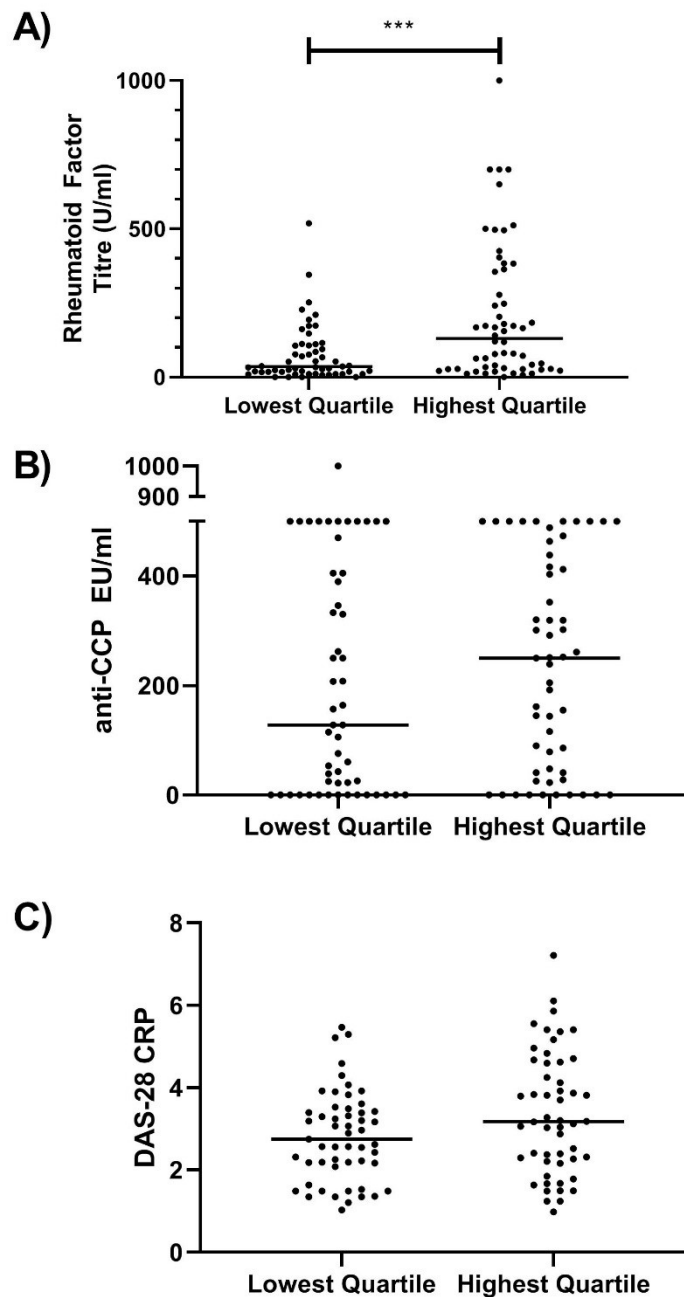


Figure 4-1 Rheumatoid factor titre, but not anti-CCP titre of DAS-28 score, is significantly higher in RA patients with a higher urinary cadmium level.

The lowest and highest quartiles of urinary cadmium level in a cohort of 200 RA patients were compared to one another and rheumatoid factor titre, anti-CCP titre and DAS-28 scores were compared between the two groups. Mean rheumatoid factor levels were significantly higher in

the highest Cd-level quartile of RA patients, although DAS-28 and anti-CCP levels were not statistically significantly different between these two groups. .Statistical significance was tested by Mann-Witney test. *** = $p < 0.005$

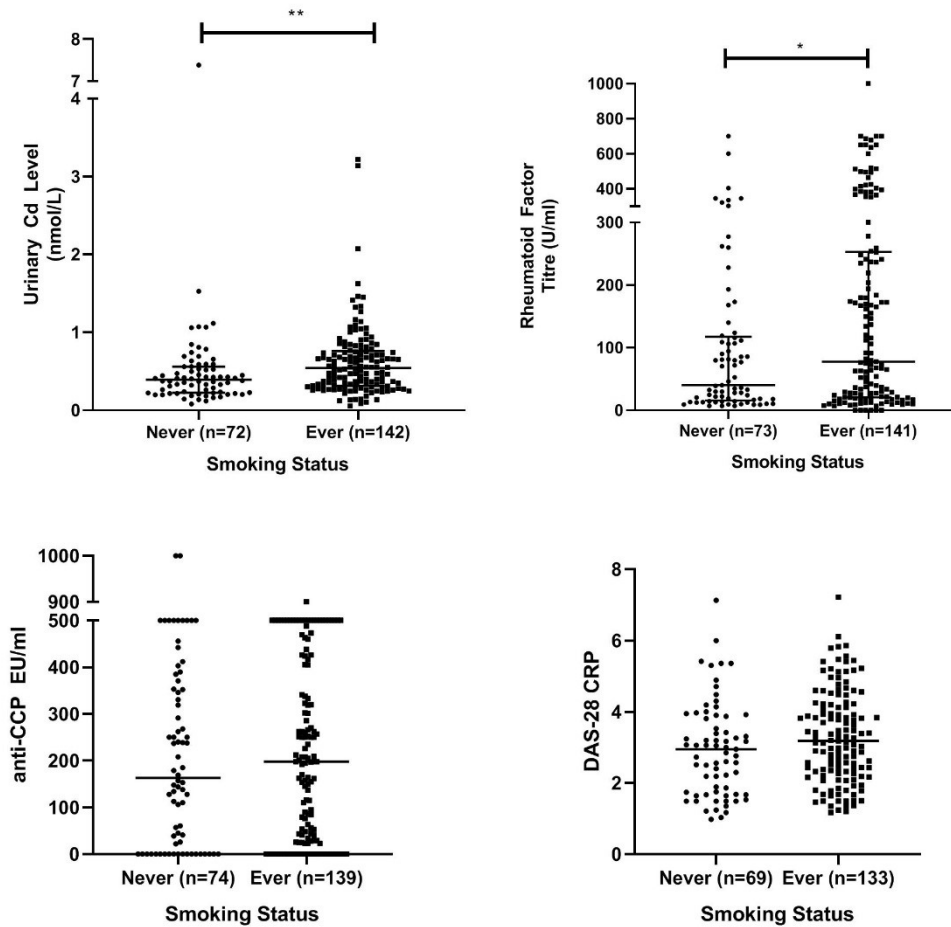


Figure 4-2 Cadmium levels and rheumatoid factor levels are significantly elevated in RA ever smokers vs RA never smokers, but not disease activity or anti-CCP titres.

The cohort of RA patients was separated into never smokers and ever smokers, and Mann-Whitney tests were used to test for statistical significance between RF, urinary Cd level, anti-CCP and DAS-28 levels. RF and urinary Cd levels in ever smoking cohort were significantly higher than the never smoking cohort.

* = $p < 0.05$, ** = $p < 0.01$. Error bars are displayed as Median \pm IQR.

Disease activity scores and anti-CCP antibody titres were not significantly different between the two groups ($p=0.9943$ and $p=0.5946$, respectively). Once again, Spearman's rank correlation was employed to test differences between cadmium level and smoking. Spearman's rank correlation coefficients and the corresponding p values are shown in **Table 4.2**. In the never smoking cohort, urinary cadmium level statistically significantly correlated weakly with anti-CCP levels ($r = 0.2586$, $p=0.0272$).

No statistical significance was observed between urinary cadmium levels, and RF and DAS-28 levels in the never smoking cohort. In the ever smoking cohort however, a different trend was observed. Urinary cadmium levels significantly correlated with RF titre ($r=0.2279$, $p=0.0066$), but not anti-CCP or DAS-28. After having examined this cohort of RA patients to establish any potential relationship between cadmium on RA patient outcomes, anti-immunoglobulin antibodies in the form of RF titres appear to correlate with urinary cadmium level.

4.3 Experimental work

Having first looked at the effect of cadmium directly on neutrophil function, it was decided to replicate these functional assays with the serum of specifically selected RA patients with varying degrees of RA severity. Given the association between elevated Cd and RF in RA smokers, it was decided to see if serum factors in these patients altered neutrophil function. Eight RA patients were chosen that had high or low levels of the following parameters: urinary cadmium level, rheumatoid factor, anti-CCP or DAS-28. In addition to this, four healthy donors were used to act as a non-disease control. The full clinical data for these 12 individuals can be found in **Table 4.3**.

	Average Urinary Cadmium Level (nmol/L)	Sex (% Female)	Rheumatoid Factor (U/ml)	Anti-CCP (U/ml)	DAS-28 Score
Never Smokers	0.5363	r value	0.1176	0.2586	0.0464
		p value	0.2161	0.0272*	0.7050
Ever Smokers	0.6191	r value	0.2296	0.0073	0.1434
		p value	0.0038**	0.9305	0.0959

Table 4-2 Urinary cadmium levels in RA patients correlate with RF titres in ever smokers.

A cohort of RA patients was separated into never and ever smoking groups. Correlations between serological markers and urinary Cd level were analysed using Spearman's rank correlation test. In the ever smoking group, urinary Cd levels correlated weakly positively. * = $p < 0.05$ ** = $p < 0.01$

Serum ID	Age (Years)	Sex	Smoking Status	RF (U/ml)	Anti-CCP (U/ml)	DAS-28	Urinary Cadmium Level (nM/L)	Sample identifier
RA73	76	M	Ex	0	0	3.42	0.244	Lowest CCP
RA84	63	M	Never	36.2	1000	1.21	0.278	Highest CCP
RA86	57	M	Ex	247.8	155	7.219	0.773	Highest DAS-28
RA125	78	F	Never	28	500	0.98	1.065	Lowest DAS-28
RA138	81	M	Ex	8.1	405.4	1.36	0.054	Lowest Cd
RA160	68	M	Current	0	53.4	2.57	0.217	Lowest RF
RA165	67	F	Never	81.6	291.5	3.87	7.384	Highest Cd
RA188	61	M	Current	700	416.9	4.12	1.044	Highest RF
HC10	40	F	Ex	N/A	N/A	N/A	Not measured	Random control
HC31	66	M	Ex	N/A	N/A	N/A	Not measured	Random control
HC104	63	F	Ex	N/A	N/A	N/A	Not measured	Random control
HC133	81	M	Ex	N/A	N/A	N/A	Not measured	Random control

Table 4-3 Clinical information regarding samples used for neutrophil functional assays.

The serum of eight rheumatoid arthritis patients and four healthy control patients was selected for neutrophil functional assays. The RA patients were selected as a result of a key serological identifier as shown in the right hand column.

Serum components are known to alter neutrophil function (360). Selected sera were first used to test their effect on altering the respiratory burst in neutrophils. Neutrophils were isolated from healthy controls, resuspended in HBSS and incubated with DHR123 in addition to a 1:10 dilution of sera from the chosen individuals as described in **Table 4.3**. In addition, autologous donor serum was used as a control. In these preliminary data, none of the serum samples tested induced ROS production in isolated primary neutrophils (**Figure 4.3**). To ensure the assay was working correctly, neutrophils were stimulated with a potent ROS agonist – 100 nM PMA, which increased fluorescence 100-fold above non-PMA treated neutrophils.

Next, the ability of patient sera to induce calcium flux in healthy isolated neutrophils was investigated. The first step was to examine calcium flux in neutrophils following a similar format to the experiments conducted with cadmium (**Section 3.4**). Primary neutrophils were isolated from whole blood and incubated with the calcium dye fluo-4-AM diluted in HBSS for 1 hour at 37°C. After incubation, baseline calcium level was measured by flow cytometry before adding an aliquot of serum (final dilution - 1:10) to neutrophils and measuring the change in calcium above basal levels in each sample.

Sera from two of the RA patients did induce statistically significant calcium release in neutrophils (**Figure 4.4**). These were RA84 ($p=0.0271$) and RA86 ($p=0.0289$), which had the highest anti-CCP titre and highest DAS-28 score respectively.

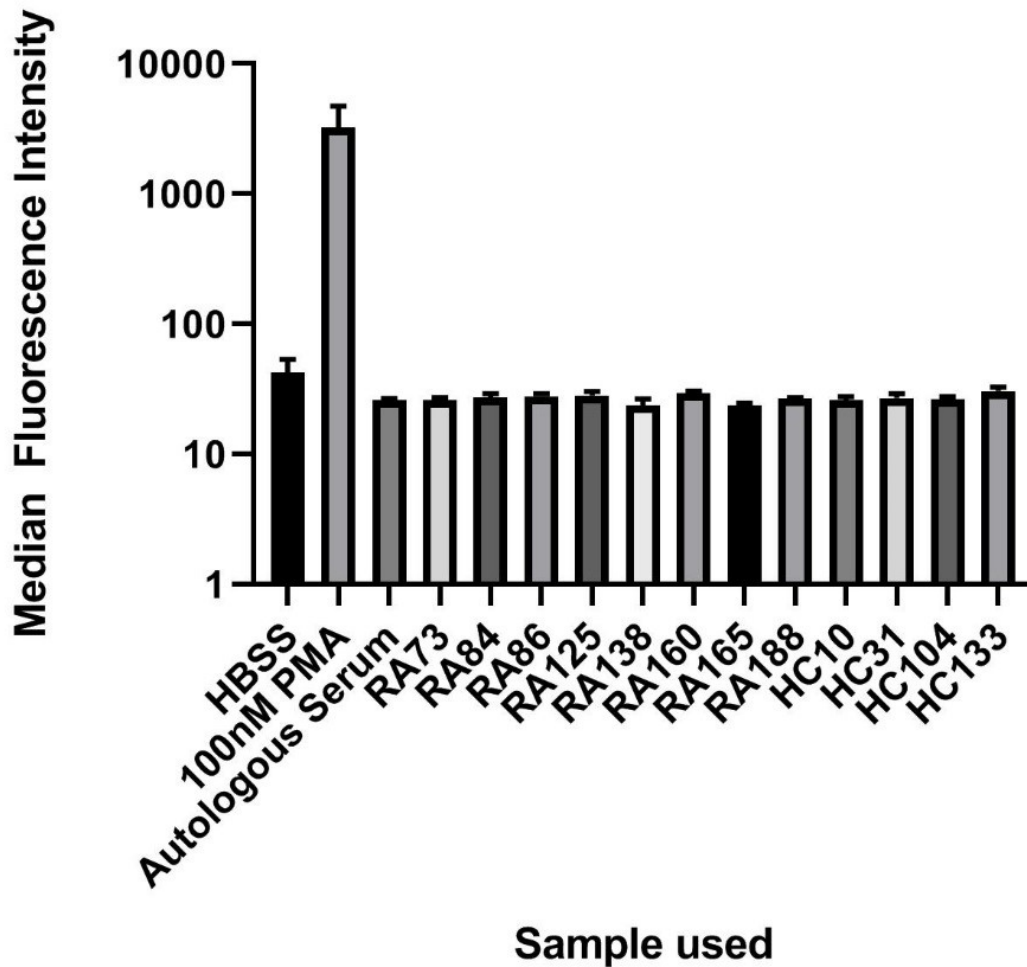


Figure 4-3 Serum from RA patients or healthy donors does not induce production of reactive oxygen species in primary neutrophils.

Primary neutrophils were isolated and treated with a 1:10 dilution of serum sample. No increase in fluorescence was observed upon serum treatment, suggesting that neither healthy or RA patient serum induces the respiratory burst in neutrophils. Data values are shown as average MFI \pm SD of two independent experiments.

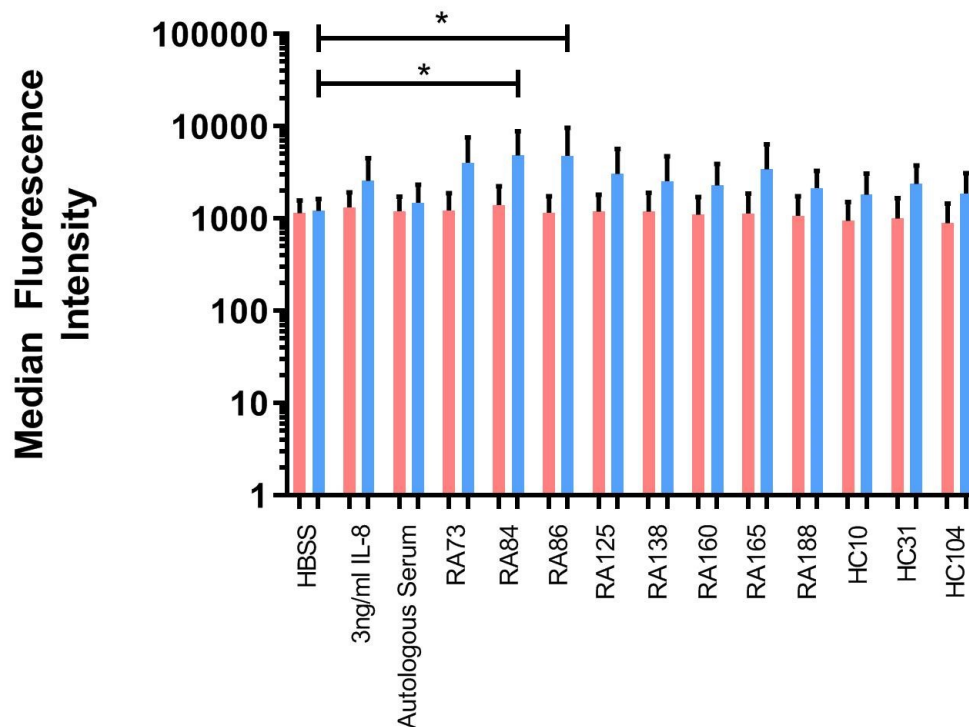


Figure 4-4 Serum from RA patients with high disease activity or high anti-CCP titre induced significant increases in baseline calcium level.

Neutrophils were incubated with the calcium dye fluo-4-AM for 1 hour before measurement of baseline cellular calcium level via flow cytometry. After this initial measurement, serum samples were added to cells at a 1:10 dilution and the calcium level was measured once more. Of all the serum samples tested, RA84 and RA86 (highest anti-CCP serum and highest DAS-28 serum, respectively), were the only samples that significantly increased calcium levels compared to unstimulated (HBSS) neutrophils. Statistical analysis tested for significance by RM two-way ANOVA with Tukey's multiple comparisons test. Data are shown as mean MFI \pm SD over two independent experiments. * = $p < 0.05$.

Statistical significance was tested via use of within sample two-way ANOVA with Tukey's multiple comparisons test. Noting minimal and isolated changes in neutrophil ROS and calcium flux changes upon serum exposure across all serum samples tested, it was investigated further to establish if RA serum was capable of inducing NETosis in neutrophils, as literature demonstrates a high propensity of RA patient neutrophils to undergo NETosis (249, 361). In RA patients, this is thought to be driven by RF immunoglobulin complexes in the form of IgA or IgM bound to IgG (205). With this cohort of varying degrees of disease severity, and serology, an investigation into if NETosis was being induced by components of RA patient serum was conducted, and further, if clinical serology from the sera used to alter healthy neutrophil function correlated with the extent of induced DNA released observed.

HL60 derived neutrophils were seeded onto 96-well plates and allowed to adhere to the bottom of the microplate. Post incubation, a 1:10 final dilution of either healthy serum or RA patient serum was added to the cells, but also in 'cell free' wells to measure the quantity of any contaminant DNA in each serum sample. After a 3h incubation, SYTOX green was added to quantify extracellular fluorescence and indirectly confirm NETosis. After quantification, cell-free serum sample RFUs were subtracted from the triplicate serum sample RFUs and then blank corrected.

Unexpectedly, the healthy control sera, rather than the RA patient sera induced statistically significant increases in release of extracellular DNA from HL60 cells compared to non-sera treated cells ($p = 0.0115$) (**Figure 4.5**). Moreover, when compared to one another, sera from healthy subjects appeared to induce more NETosis than RA sera ($p = 0.0002$).

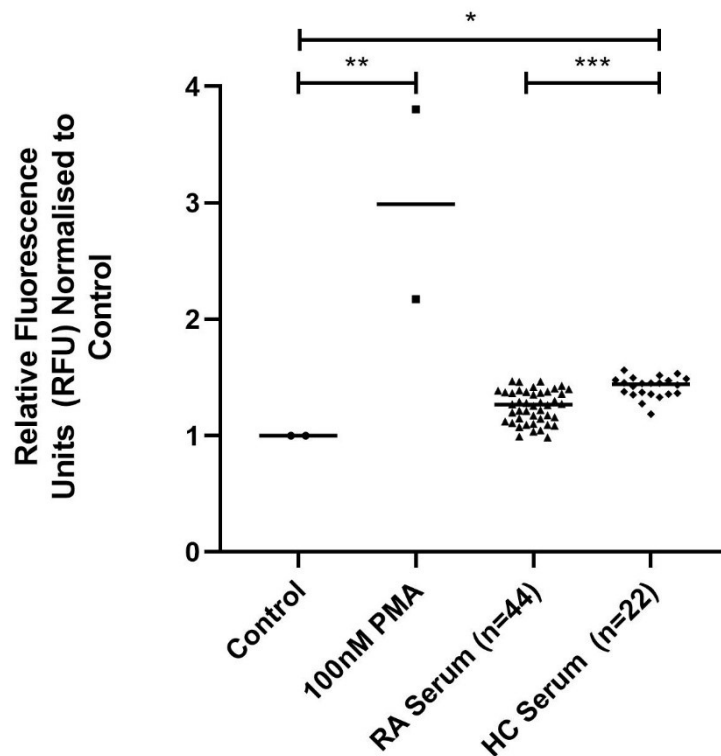


Figure 4-5 Healthy control serum significantly increases extracellular DNA, but not RA serum in HL60 derived neutrophils.

HL60 derived neutrophils were seeded onto a 96 well plate and treated with a 1:10 dilution of either RA patient or healthy control serum. After 3h incubation extracellular DNA was measured by SYTOX green addition. The serum of healthy control patients significantly increased extracellular DNA ($p=0.0293$) compared to no significant increase with RA serum ($p=0.5680$) when statistically analysed to the control cells. The increase in DNA between the healthy serum and RA serum was also statistically significant ($p=0.0002$). Statistical analysis was performed using a Kruskal-Wallis test with Dunn's post-test. Data are shown as the median relative fluorescence units as a normalised unit to unstimulated cells. * = $p<0.05$, ** = $p<0.001$, *** = $p<0.005$. Data are derived from two independent experiments.

Nevertheless, a correlatelon was calculated between the degree of extracellular DNA release induced by RA sera with clinical serological data to discern if RF had any influence of the degree of DNA release occurring in the HL60-derived neutrophils. Spearman's rank test was used to test if the quantity of DNA release correlated to RF, anti-CCP or DAS28 values (**Figure 4.6**). Rheumatoid factor ($r=0.03214$, $p=0.8359$), anti-CCP ($r=0.03478$, $p=0.8227$) and DAS-28 ($r=-0.1421$, $p=0.3756$) showed no correlation with DNA release from HL60 cells using RA patient serum. This indicates that it is unlikely that RA patient serum induces NETosis in HL60-derived neutrophils, and any increases in extracellular DNA because of RA sera addition are not related to any influencing factor or RA serological markers. Alternatively suppressive factors in sera may have influenced the results of the assay.

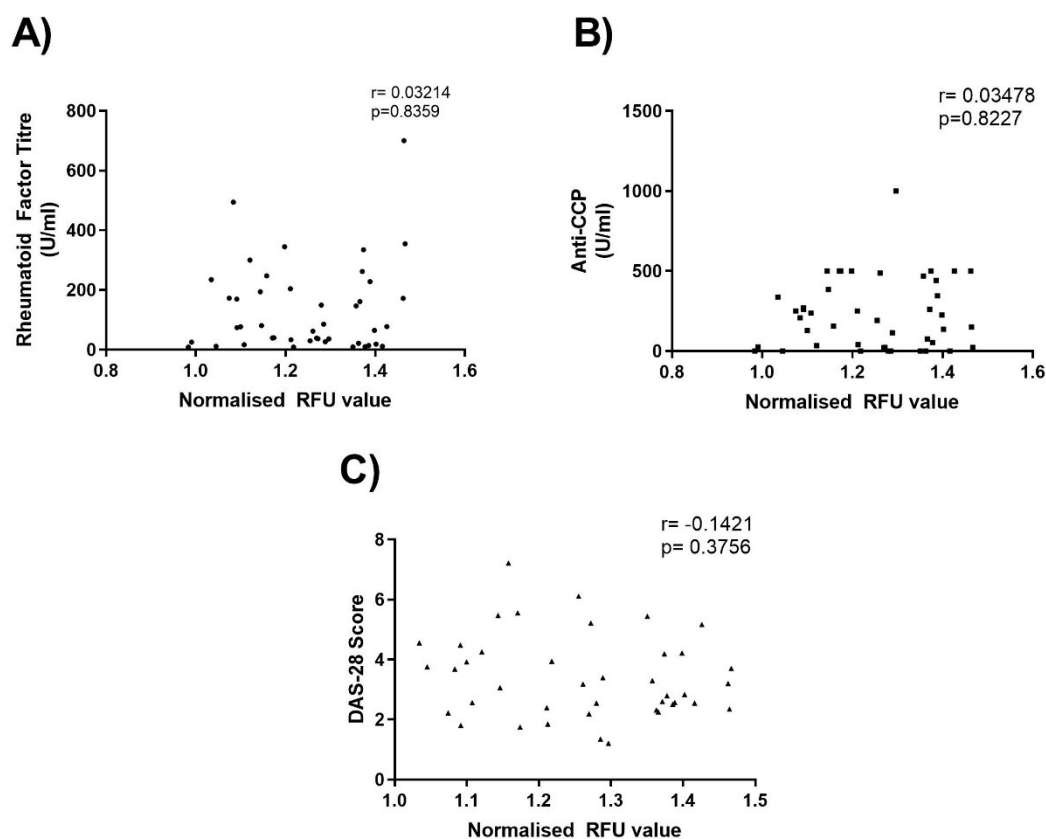


Figure 4-6 The degree of extracellular DNA release induced by rheumatoid arthritis patient serum does not correlate with any of the clinical serological markers used to monitor RA progression.

The RFUs of the RA serum (n=44) from the SYTOX green assay were normalised to the untreated RFUs. These values were then compared to A) rheumatoid factor, B) anti-CCP and C) DAS-28. Statistical analysis used spearman's rank correlation coefficient to test for statistically significant correlations. None of the clinical markers significantly correlated with the normalised RFU values. Each graph displays both the r value and p value of each comparison.

4.4 Discussion

In this section, it has been shown that urinary cadmium levels correlate with RF titres in ever-smokers. In this cohort of 200+ RA patients, that in the context of urinary cadmium levels, the highest quartile has significantly higher RF titres than the lowest quartile. Studies investigating the relationship between RA and cadmium are relatively novel, with Joo and colleagues providing the only real additional evidence that such a relationship exists (315). This stresses the novelty of this finding, and the correlations found within it may support the hypotheses posed by this research group (321, 362, 363).

Examination into the effect of other heavy metals in RA populations is also rather scant. Irfan *et al.* measured the levels of lead, cadmium, nickel and chromium in RA patients in Pakistan and found significant increases in these serum levels compared to control patients (n=100 for both) (364). In terms of why these metals may be influencing RA pathology, Goldberg *et al.* found that silver, mercury and lead negatively affected collagen synthesis in human synovial cells, and thus may exacerbate the rate at which the synovium is degraded compared to patients where lower levels of heavy metals are present (365).

In a more general sense however, it appears that in healthy isolated neutrophils, that the serum of RA patients does not contain any abnormal proteins that may evoke a dysfunctional response. In RA patients, neutrophils are shown to display irregular responses. In synovial fluid, RA patient neutrophils spontaneously release high levels of ROS (366). Cell-free synovial fluid from RA patients, and to a lesser extent, serum, also induce the respiratory burst in healthy neutrophils (367). The diminished response seen by serum

found to increase respiratory burst activity to roughly 175% (367). This study pooled the serum samples of 7 patients however, citing high variability.

This may well mean that the reason for the lack of response found by the respiratory burst experiments could be attributed to insufficient immunostimulatory molecules in each serum sample. The aim was to determine if patient serum with varying degrees of disease severity induced differing levels of neutrophil stimulation. As insufficient responses were observed from single serum samples, the data suggests that no single component of RA patient serum modulates the neutrophil respiratory burst in healthy neutrophils. This contradicts the findings of Chowdhury *et al*, who found that serum from RA patients induced ROS production in healthy neutrophils (368). The differences between these results could be due to methodology. The authors do not state serum dilution used in these experiments, nor do they state if serum was pooled during these assays. Given prior literature regarding RA serum and the respiratory burst in neutrophils, it may well be that the serum samples from RA patients were pooled together. The artificial experimental system in itself has flaws, in that these experiments were healthy neutrophils exposed to RA serum for the first time, in contrast to certain reports in the literature where RA neutrophils are exposed to autologous RA serum. The differences may be explained to the fact that it is constant exposure to these serum elements that may drive dysfunctional responses.

Neutrophils in RA patients have impaired calcium signalling when activated by Fc gamma receptors (369). Other cells in RA, such as T lymphocytes also show reduced levels of calcium release upon stimulation (370). This suggests that cellular dysfunction in RA leucocytes may be somewhat influenced by reduced calcium signalling. Rheumatoid factor cross-links with membrane bound IgG on

neutrophils and results in increases in cytosolic calcium levels via activation of the FcγRIIIb receptor (371, 372). This would suggest that in the calcium flux experiments, that the RA serum with high levels of RF could induce flux in healthy neutrophils. In all the serum samples used, the only two samples that resulted in increases in cytosolic calcium levels were RA84 and RA86. If RF was capable of increasing calcium levels in neutrophils, then calcium levels should have increased upon addition of serum samples RA165 and RA188, which had RF titres greater than these. The other potential causative agent for these cytosolic increases may be anti-citrullinated protein antibodies. RA84 contained by far the highest ACPA titre, yet small levels of RF. The paradoxical positive increases seen by RA84 and RA86 but not RA165 and RA188 may suggest that neither RF nor anti-CCP increases cytosolic calcium levels in healthy neutrophils. It may well be that the responses observed in these samples are driven by other components in the serum, such as cytokines, which were not accounted for in these experiments. It would be of great interest to examine the response in neutrophils to synovial fluid of these patients, as synovial fluid is a more direct reflection of the inflammatory environment in RA. Furthermore, synovial fluid in RA patients inhibits neutrophil apoptosis, which could result in dysfunction through prolonged overactivation (246). It would also be a good opportunity to examine the function of RA patient neutrophils in the above assays used in order to correlate the responses with clinical parameters.

Neutrophil extracellular trap formation has previously been shown by Chowdhury and colleagues to occur upon stimulation of healthy neutrophils with RA patient serum and synovial fluid (368). In this study, healthy neutrophils were isolated from donors and incubated with RA patient serum or synovial fluid, or healthy donor serum (368). The authors found via

immunohistochemistry that RA serum, and to a greater extent, RA synovial fluid, induced NETosis in neutrophils (368). Issues with reporting NETosis via immunohistochemistry have been previously reported. The authors also used a sandwich ELISA targeting myeloperoxidase and cellular DNA, to confirm NETosis in neutrophils. In RA patient serum samples, higher levels of these NET remnants were present.

Nevertheless, the results found by these experiments through usage of the SYTOX green assay showed no NETosis from incubation of HL60-derived neutrophils with a 1:10 dilution of RA patient serum. It may be that a 1:10 dilution is too dilute to elicit cellular changes, and so higher concentrations may initiate cellular responses not seen in assays. Intriguingly, in these experiments, healthy control serum resulted in a greater amount of extracellular DNA release. This could suggest that RA serum may contain factors that are capable of suppressing NETosis, as opposed to inducing it (112, 373-375).

4.5 Conclusion

In a cohort of patients with varying degrees of disease severity, smoking status and urinary cadmium level, it appears that cadmium may influence RF titres. Urinary cadmium levels were significantly higher in a subpopulation of this cohort with a history of smoking compared to a subgroup that had never smoked. In addition to this, urinary cadmium levels correlated with RF titres in this subgroup, suggesting that cadmium may play a role in the formation of the rheumatoid factor autoantibody complex in smokers.

Overall, it appears that RA patient serum does not induce any noticeable response in either isolated primary neutrophils or HL60-derived neutrophils. The

respiratory burst is unaffected by RA patient serum, despite literature showing strong evidence for this. Explanations for these differences may be due to lack of pooling the RA serum. Some samples have shown to induce calcium release in primary neutrophils however, the unique serological marker for these samples (highest anti-CCP titre and DAS-28 score, respectively) is not highlighted in the literature as a potential reason for increases in cytosolic calcium levels. Similarly, RA patient serum non-significantly increases extracellular DNA levels, though it appears that healthy control serum increases these levels to a significant level, suggesting a potential suppressive effect in the RA serum samples in this cohort.

5 Modifications to IgG as a potential link to rheumatoid arthritis

5.1 Introduction

Having noted the potential enhancing effect of cadmium on NADPH-oxidase mediated processes (Chapter 3) and subsequently finding that RF titres of RA patients are higher in patients with higher cadmium levels (Chapter 4), a retrospective approach to analyse RA patient sera was used in order to look for any relevant protein modifications. Having noted the potential role of cadmium in RA at both ends of the disease spectrum (initiating and end-point) via calcium flux and the respiratory burst, other possible triggers of RA disease pathology that remain unanswered were investigated. The objective of this chapter were to:

- Use mass spectrometry to precisely identify protein modifications in RA patient serum previously shown to contain citrullinated proteins via western blotting
- Given the relevance to RA, focus specifically on modifications to IgG that are likely to be highly prevalent in RA patient sera
- Establish an in-house capture ELISA that could detect patient antibody binding to modified and unmodified variants of Fc fragments, to help confirm mass spectrometry findings

5.2 Cohort Serology

During this project, access to a biobank of sera from RA patients with (n=50, RA) and without bronchiectasis (n=52, BRRA), and bronchiectasis (n=122, BR) alone patients was available. Further information on this cohort is shown in **Table 5.1.**

	Median Age (IQR)	Sex (% Female)	Smoking Status (Ever %)	Median Rheumatoid Factor (U/ml) (IQR)	Median Anti-CCP (U/ml) (IQR)	Median DAS-28 Score
RA Only (n=50)	65.61 (14.8)	36.72	56	21.95 (60)	5.75 (243)	2.590
BR Only (n=122)	49.97 (26.7)	61.2	39.3	10.40 (7)	1.2 (1.2)	N/A
BRRRA (n=52)	65.98 (12.8)	71.1	42.3	65.00 (107)	195 (293)	3.51
Healthy Control (n=78)	60 (17)	74	42.4	<7.00	1	N/A

Table 5-4 Patient and disease control demographic for the second cohort of serum samples used.

Abbreviations: DAS-28 = Disease Activity Score in 28 joints tested, IQR = Interquartile range. This figure has been adapted from the work of Chikazawa et al. (216).

As previously stated in **Section 1.4.2**, up to 30% of BR patients may develop RA, and the mechanisms through which this seroconversion occurs is unknown (259). This cohort has been reported on previously, with the sample collection being undertaken by Dr Liz Perry (277, 282, 283). Serological measurements on this cohort have been previously published, with the most significant findings being related to the differences between BRRA patients and RA patients alone. The RA cohort was specifically chosen for having no history of lung disease, as opposed to the BRRA cohort, where 58% had bronchiectasis before onset of RA symptoms. In general, the BRRA cohort presented with greater disease activity scores and higher anti-CCP and RF positivity than the RA only patients (283). Smoking was not a confounder for any of the serological findings.

This cohort has previously been used to investigate the role of anti-calreticulin and anti-citrullinated calreticulin antibodies as a prognostic biomarker for double seronegative (RF-negative and anti-CCP negative) RA patients, published in 2017 (216). More recently, this cohort was used to publish findings that the sera of patients with BRRA have increased levels of Galectin-9, and that Galectin-9 can induce PAD4 activation in neutrophils (376).

Subsequent to the 2017 study, four of the BR-only patients developed RA 12-18 months after serum sample collection (herein referred to as BR_{sero}). It was of great interest to ascertain if the serum characteristics of these four individuals differed from other BR patients. In the BR-only cohort, these four individuals tested positive for both RF and ACPAs at the time of sampling and were the only subjects in the 122 sample cohort to do so.

Given the positivity for antibodies and immune complexes in these particular samples, the proteomic profile of the immunoglobulins was analysed to discern

if any differences were present between BRRA, RA and healthy serum, and also the serum of these four individuals.

The ultimate aim of this chapter was to explore this valuable cohort of RA patients on a proteomic level via usage of mass spectrometry.

5.3 Western blotting for citrullinated proteins

As explained previously in **Section 1.4.1.3**, in this cohort of patients, antibodies to citrullinated calreticulin were observed in 49% of RA/BRRA/BR_{sero} (collectively referred to as RA_{any}) samples, but not healthy control samples (216). Following on from prior work detecting these antibodies in the serum, the presence of citrullinated calreticulin in these serum samples was examined to determine if serum citCRT was expressed at higher levels in RA_{any} patients than healthy donors.

Representative serum samples from the different patient/control groups were selected for immunoblotting; two healthy controls and BR patients, two seroconverted BR samples and four RA and BRRA serum. In brief, samples were run on a 8-16% Tris/Glycine/SDS gradient gel and transferred onto a nitrocellulose membrane via semi-dry transfer with the Bio-Rad turboblot (**Section 2.1.2.**) The blot was probed for citrullinated protein with an anti-citrulline antibody (ab100932, Abcam, UK), followed by a near infrared secondary ab and then imaged on a LiCor scanner. Citrullinated protein was present in high levels in all bar one (lane 10) of the ten RA_{any} serum samples (lanes 6-9, 11-15), but not the healthy control or BR only samples (lanes 1, 2, 4 and 5) (**Figure 5.1**).

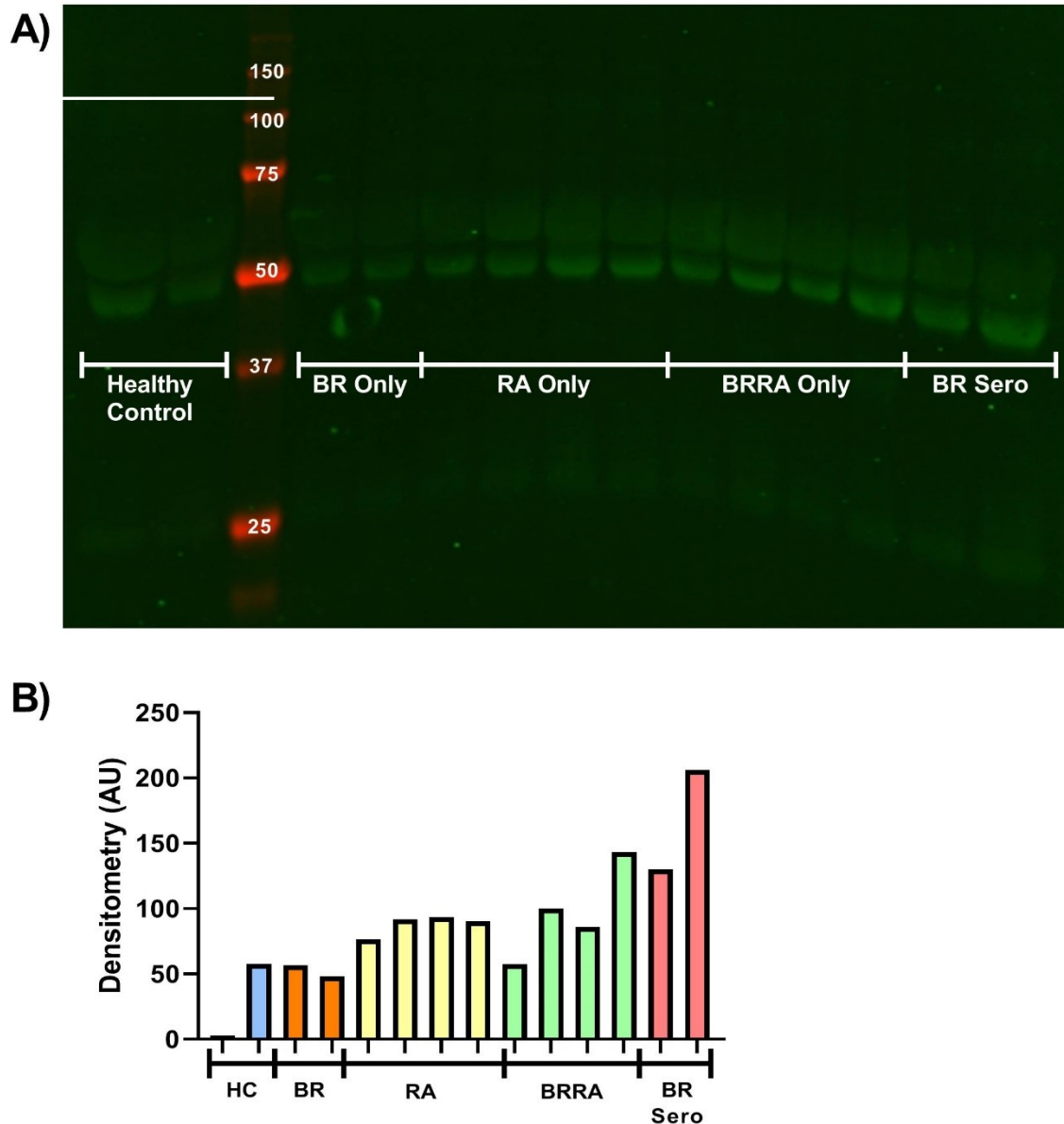


Figure 5-1 Citrullinated protein is present in the serum of patients with rheumatoid arthritis, bronchiectasis with rheumatoid Arthritis, and seroconverted bronchiectasis patients.

A) Serum samples were loaded onto a commercial Tris/Glycine/SDS 8-16% gradient gel and SDS-PAGE and immunoblot performed. The membrane was probed for citrullinated protein. B) After imaging the membrane, densitometry was used to measure the intensity of citrullinated protein in each sample. The cohort of each sample is listed below the graph.

Densitometry was used to quantify band at the 50kDa region The RA_{any} samples contained roughly 1.5-4 times the quantity of citrullinated protein compared to the healthy and BR only samples. Mass spectrometry was then used to identify the citrullinated protein species within this band.

5.4 Proteomic analysis

A selection of samples were chosen for further proteomic analysis from the cohort shown in **Table 5.1**; four RA, BR and BRRA, and 10 healthy control serum samples to be probed via mass spectrometry. The demographics for these samples are listed in **Table 5.2**. Samples were run according to the same method shown above (Section 5.2). Once the gel run had completed, proteins were then visualised with Coomassie blue staining. Bands were excised around the 50kDa size range, as this was where the majority of citrullinated protein in each sample was detected (**Figure 5.2**). These bands were then tryptic digested and protein identification revealed by mass spectrometry.

As discussed in **Section 2.4.3**, proteins identified were scrutinised for peptide sequences containing citrullinated arginines, or carbamylated lysines using the Proteome Discoverer 1.4 software. The choice for these two amino acid modifications pertains to the nature of autoantibodies found in RA. Citrullination and carbamylation are PTMs through which ACPAs and Anti CarPs form, respectively.

ID	Cohort	Age	Gender	Smoking	Anti-CCP (U/ml)	ACR Anti-CCP Interpretation	RF Result (U/ml)	ACR RF Interpretation
1	BR*	72	F	Never	522	Very High Positive	56.2	Low Positive
2	BR*	67	F	Ex	43	High Positive	16	Low Positive
3	BR*	78	M	Never	340	Very High Positive	22.00	Low Positive
4	BR*	72	F	Ex	92	High Positive	130.00	Very High Positive
5	BRR	76	F	Never	88	High Positive	27.5	Low Positive
6	BRR	81	F	Never	600	Very High Positive	130	Very High Positive
7	BRR	60	F	Never	229	High Positive	130	Very High Positive
8	BRR	66	F	Never	340	Very High Positive	130	Very High Positive
9	RA	53	F	Current	197	High Positive	74.6	High Positive
10	RA	39	F	Current	6.9	Negative	78.1	High Positive
11	RA	44	F	Current	340	Very High Positive	130	Very High Positive
12	RA	62	F	Current	1.5	Negative	8.4	Negative
13	Control	53	F	Never	1	Negative	0	Negative
14	Control	80	F	Never	1	Negative	0	Negative
15	Control	68	F	Never	1	Negative	0	Negative
16	Control	40	F	Never	1	Negative	0	Negative
17	Control	80	F	Never	1	Negative	0	Negative
18	Control	37	F	Current	1	Negative	0	Negative
19	Control	54	F	Current	1	Negative	0	Negative
20	Control	52	F	Current	1	Negative	0	Negative
21	Control	81	M	Ex	1	Negative	0	Negative
22	Control	56	F	Current	1	Negative	0	Negative

Table 5-2 Demographics of individual subjects including age, gender, smoking status, anti-CCP and RF test result and interpretations.

ID: identifier; CCP: cyclic citrullinated peptide; RF: rheumatoid factor; ACR: American College of Rheumatology; BR: bronchiectasis; RA: rheumatoid arthritis; F: female; M: male. *: bronchiectasis patients who went on to develop RA 12–18 months post-sampling. Age of the control samples is presented as median with interquartile range.

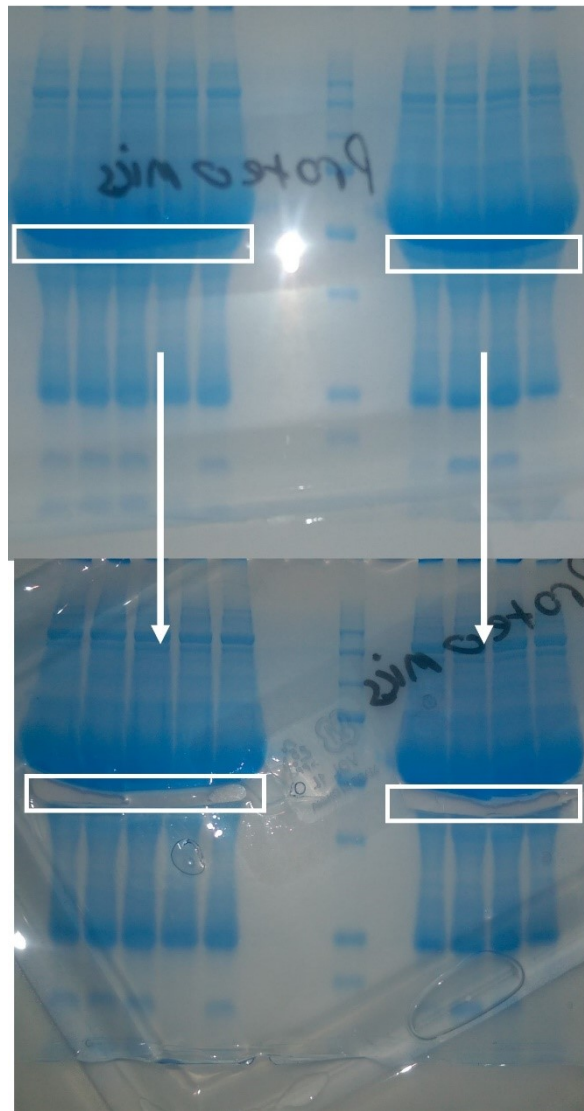


Figure 5-2 Example of serum sample bands excised for mass spectrometric analysis.

After SDS-PAGE, proteins within the gel were stained by Coomassie blue and protein bands were excised below the albumin band around 50kDa. Excision first performed between sample lanes, and then between protein bands. Bands were then analysed by mass spectrometry.

Modified peptide sequences were detected by “peptide spectrum match” (PSM) counts, which refers to the frequency with which a given peptide sequence was counted by the mass spectrometer. Across all samples test, over 300+ proteins were found to be detected in the protein bands tested ranging from 0.14-95% of full protein sequence coverage. Incidence of PTMs in these proteins varied drastically, though the two proteins found to be most abundantly modified and highly represented in these samples were alpha-1-antitrypsin (uniprot code SERPINA1) and the heavy chain of immunoglobulin G (uniprot code IGH@). Given the potential significance of these two proteins to the pathology of RA, a further set of serum samples (derived from the cohort shown in **Table 4.1**) were subsequently analysed by mass spectrometry following the same process described above. The individual serology results from these additional patient samples are shown in **Table 5.3**.

5.4.1 Alpha-1-antitrypsin modifications

Alpha-1 antitrypsin was found to be modified in all samples tested, with healthy controls and RA_{any} samples exhibiting both citrullination and carbamylation modifications (**Figures 5.3 and 5.4**). By far the most frequent posttranslational modification observed was citrullination of the arg220 residue.

This occurred in 15 of the 26 RA patient sera screened samples tested, and 5 of the 10 healthy controls, of which three were never smokers, and three were smokers (**Figure 5.3**).

RA ID	Age	Gender	Smoking	Anti-CCP Result (U/ml)	ACR Anti-CCP Interpretation	RF result (U/ml)	ACR RF Interpretation
23	74	M	Current	153.6	High+ve	635.9	High+ve
24	70	M	Never	0	Negative	7	Negative
25	65	M	Current	212	High+ve	650	High+ve
26	70	M	Ex	333.1	High+ve	518.8	High+ve
27	64	M	Current	0	Negative	650	High+ve
28	67	M	Never	268	High+ve	9	Negative
29	69	M	Current	257	High+ve	685	High+ve
30	81	M	Ex	405.4	High+ve	8.1	Negative
31	63	F	Never	403	High+ve	700	High+ve
32	80	M	Current	235	High+ve	0	Negative
33	64	F	Ex	261	High+ve	700	High+ve
34	73	F	Current	0	Negative	0	Negative
35	53	F	Never	0	Negative	8.9	Negative
36	61	M	Current	416.9	High+ve	700	High+ve

Table 5-3 Demographics of individual subjects in the Cd-RA cohort including age, gender, smoking status, anti-CCP and RF test result and interpretations.

ID: identifier; CCP: cyclic citrullinated peptide; RF: rheumatoid factor; ACR: American College of Rheumatology; BR: bronchiectasis; RA: rheumatoid arthritis; F: female; M: male.

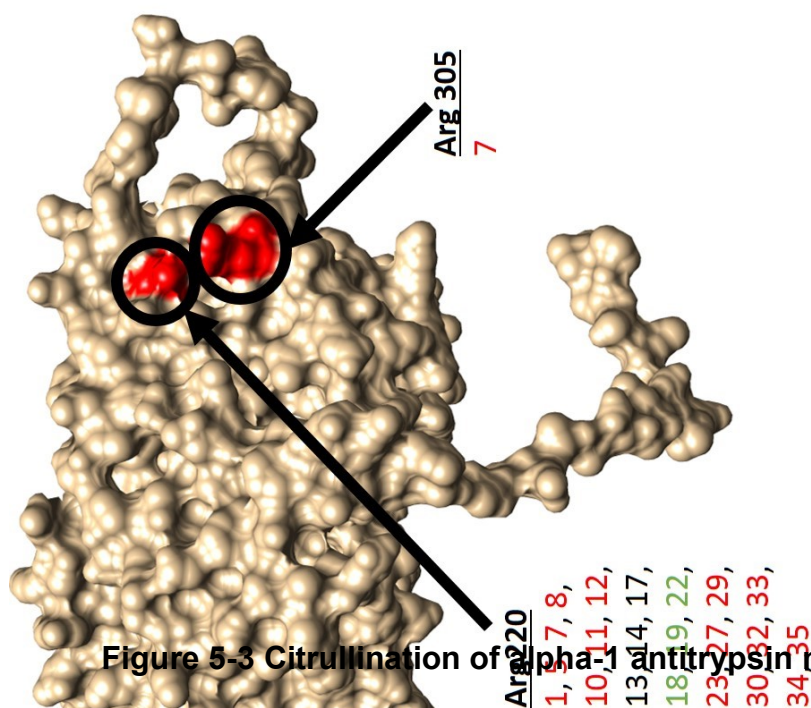
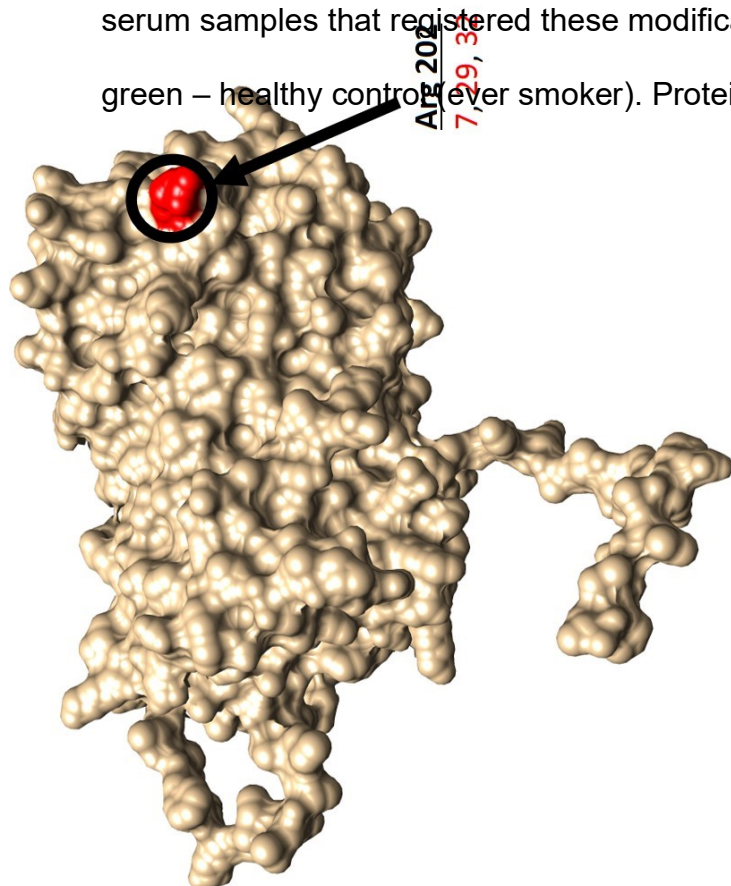


Figure 5-3 Citrullination of alpha-1 antitrypsin mainly occurs in RA patients.

The arginine sites on alpha-1 antitrypsin that registered peptide sequences that had been citrullinated were identified by mass spectrometry. Arginine 220 was by far the most commonly citrullinated amino acid. Underlying the serum samples that registered these modifications are listed as follows: red - RA_{any} patient, black - RA_{never smoker} patient, green – healthy control (never smoker). Protein modelling was performed using the UCSF Chimera.



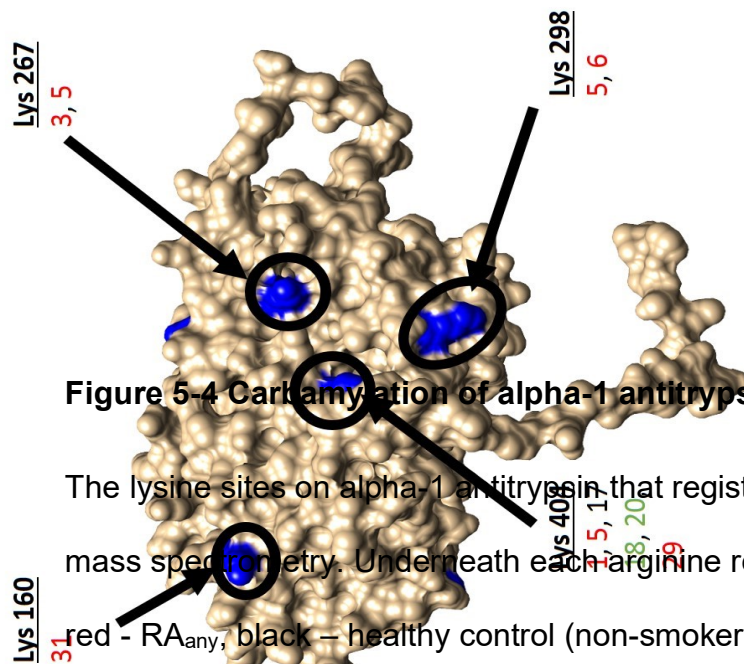
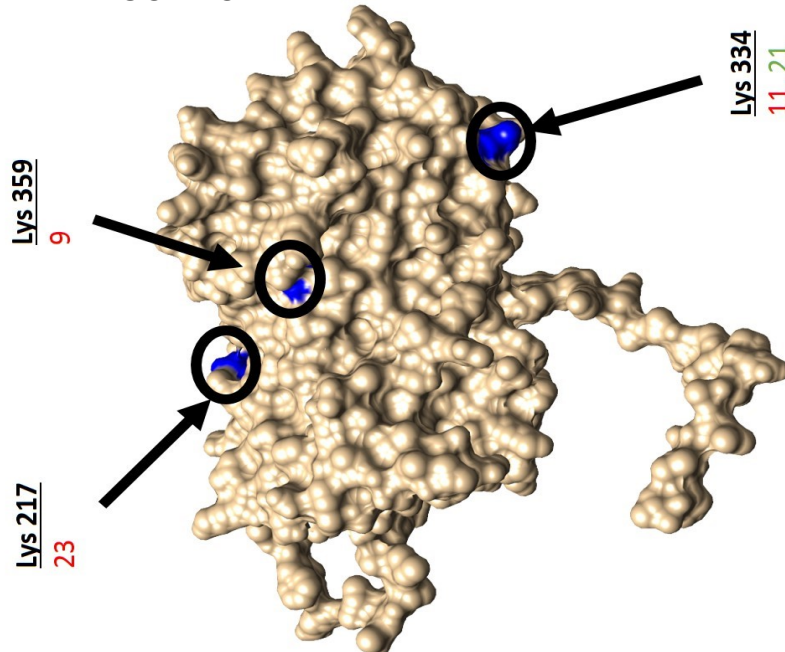


Figure 5-4 Carbamylation of alpha-1 antitrypsin mainly occurs in RA patients.

The lysine sites on alpha-1 antitrypsin that registered peptide sequences that had been mass spectrometry. Underneath each arginine residue, the serum samples that registered

red - RA_{any}, black – healthy control (non-smoker), green – healthy control (ever smoker)

UCSF Chimera software.

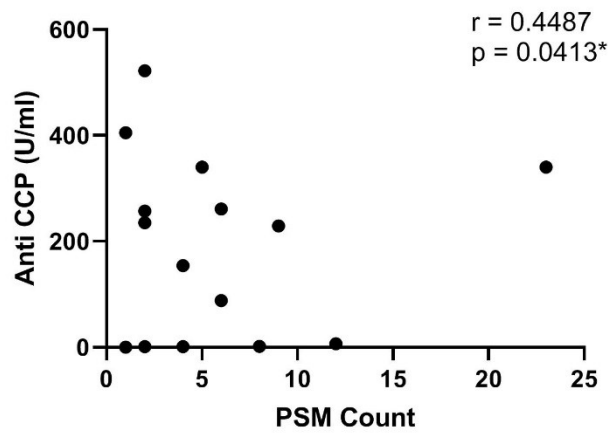


Further correlation into the frequency of arg220 citrullination was analysed statistically. The PSM count of citrullination of arg220 for each sample was compared to anti-CCP and RF titres.

The extent of citrullination of arg220 correlated significantly ($r = 0.4487$ $p = 0.0413$) with anti-CCP titres in all samples (**Fig 5.5A**). Rheumatoid factor titre showed positive correlation with PSMs, but this did not reach the significance threshold ($r = 0.3991$, $p = 0.0731$). Interestingly, the patient sample with the greatest quantity of citrullination at numerous arginine sites was a female BRRA patient who had never smoked with no single noticeable aspect of serology accounting for this increased protein citrullination; the patient's ACPA titres were positive, yet no higher than any other samples. The only distinguishing characteristic of this patient was the duration of RA of 30 years prior to bronchiectasis diagnosis.

Carbamylation modifications on various lysine residues were less frequently observed across all samples, though were seen more commonly in the RA_{any} samples or healthy control smokers. The patient sample with the relatively highest degree of lysine carbamylation was from a female BRRA never smoker with even lower serological scores than the aforementioned BRRA patient. An element of homogeneity was observed in terms of PSM counts across all samples. Of the samples that registered carbamylation of lysine across all residues detected, PSM counts for each sample ranged between 1 and 4 for each sample.

A)



B)

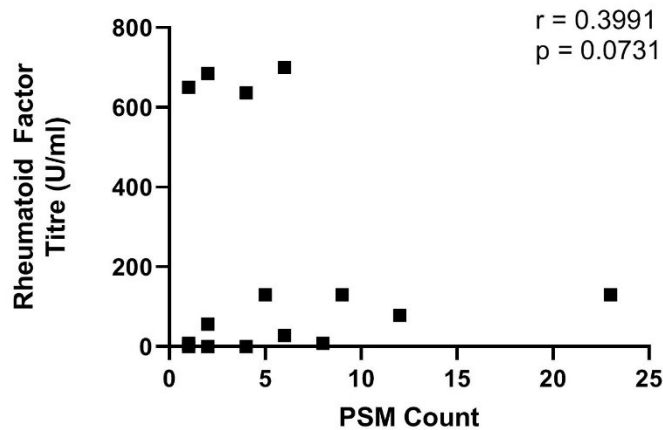


Figure 5-5 The quantity of citrullination of the amino acid residue arginine 220 on alpha-1 antitrypsin significantly correlates with anti-CCP serological scores, but not rheumatoid factor.

The number of peptide sequences containing a citrullinated arginine 220 residue across the 21 positive samples detected via mass spectrometry (PSM count). Spearman's rank correlation coefficient was used to compare PSM counts for arg220 the clinical serological markers A) anti-CCP titre and B) RF titre.

Noting the indistinguishable differences between all disease cohorts, it may be concluded that lysine carbamylation of A1-AT is not associated with RA pathology.

5.4.2 Modifications to immunoglobulin G

Various forms of the Ig heavy chain (IGH) sequence were found to be modified in the samples tested, the majority of which belonged to the IgG1-3 class.

Citrullination and carbamylation were detected across all regions of the heavy chain, with lysine carbamylation more evident than arginine citrullination (**Figure 5.6**). Of particular note, specific arginines in RA patients IgGHC appeared to be more susceptible to PTM and at a greater frequency than HC patient IgGHC.

The HC regions of IgG where modifications were found to be most concentrated in the RA_{any} samples were the amino acid sequences associated with C1q binding to IgG and the RF IgM-IgG binding site (377, 378). The locations of these sites, including the peptide sequences are shown in **Figure 5.7**.

Carbamylation occurred very commonly in these two sites in the RA_{any} samples, though citrullination of the arginine residue in the RF binding site sequence was restricted to only certain RA samples (**Table 5.3**).

Carbamylation and citrullination in other regions of the Ig heavy chain were observed in most samples irrespective of disease or healthy status, suggesting some PTMs occur naturally. The quantity of peptide sequences found to contain carbamylated lysines was found to be statistically significantly higher ($p=0.0359$) in the RA_{any} samples compared to the healthy control samples (**Figure 5.8A**). Citrullinated arginine residues were not significantly higher ($p = 0.1373$) compared to healthy control samples (**Figure 5.8B**).

To confirm the increased levels of citrullination and carbamylation in immunoglobulins as determined by mass spectrometry, sera from the mass spectrometry analysis cohort were immunoblotted and probed for anti-citrulline and carbamylation residues. Briefly, IgG was isolated from sera via FPLC and the purified IgG for carbamyl-lysine and citrulline. Immunoblotting confirmed the presence of citrullinated and carbamylated residues on this protein (**Figure 5.9**). Interestingly, despite greater quantities of carbamylated lysine being detected in the Ig compared to citrulline by mass spectrometry, immunoblotting appeared to show greater relative quantities of citrulline than carbamyl-lysine (**Figure 5.9B vs Figure 5.9D**), this might be reflective of the avidity of the commercial antibodies used for detection.

Nevertheless, having noted the presence of both carbamylated lysine and citrulline in the IgG isolates, these type of modifications to IgG were tested to see if they were responsible for the formation of RF, and if RA patient antibody-antibody complexes had antibodies formed to IgG molecules that contained these posttranslational modifications.

5.4.3 Preparation of Fc fragment isolates from pooled sera

After noting the increased presence of citrullination and carbamylation in the CH₂ RF binding region of the heavy chain of immunoglobulin G, modifications to the Fc region of IgG led to a greater recognition by RA patient antibodies were examined, as modifications to this region were found to be substantially elevated in the RA_{any} cohorts. With modifications to other regions having been observed in all samples, those specific to the Fc component of immunoglobulins were examined to see if they could be a potential driving factor in RA pathology.

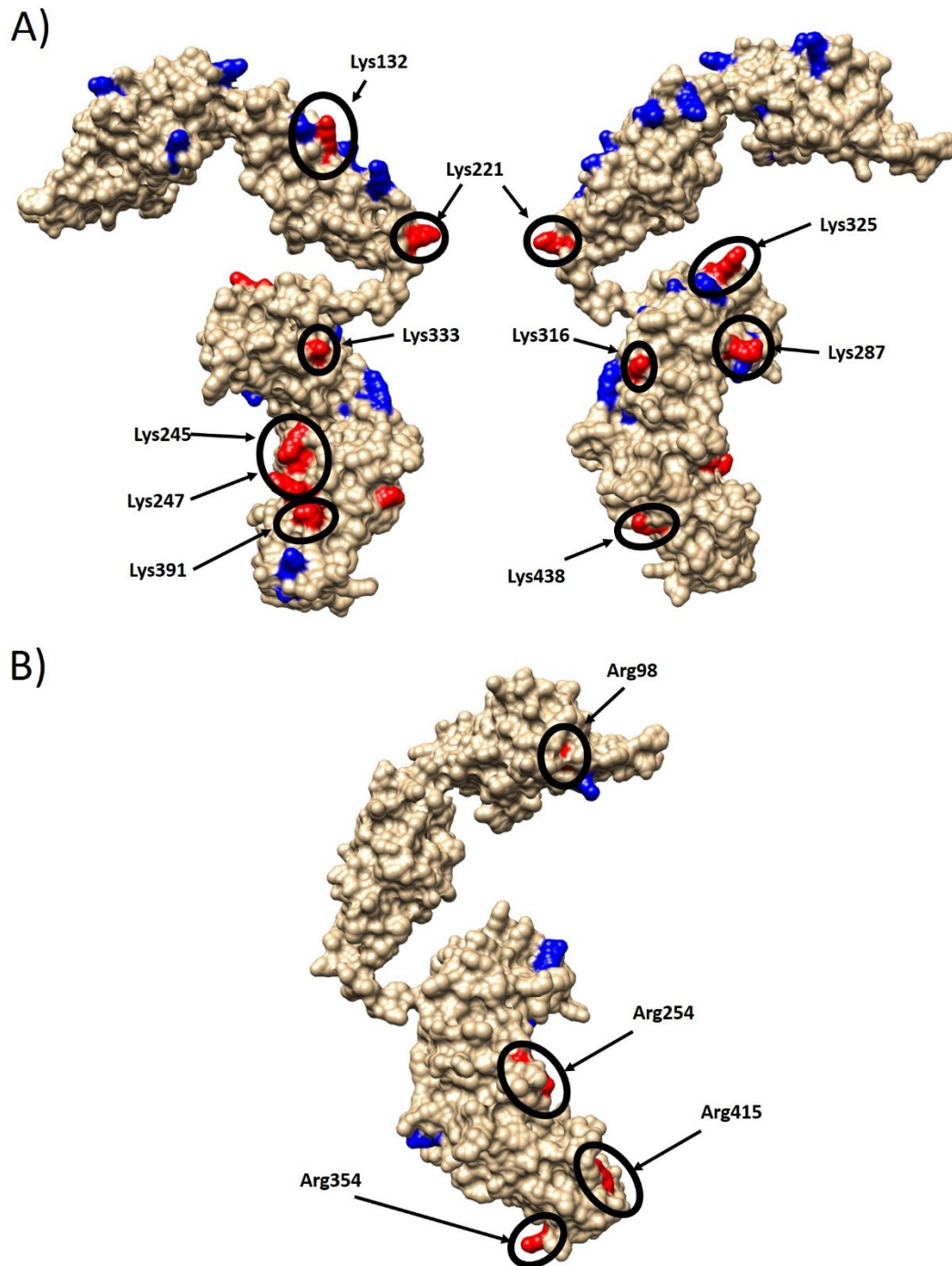


Figure 5-6 Protein modelling of the heavy chain of immunoglobulins and the modified amino acid residues detected by mass spectrometry.

A) The lysine residues on IgG heavy chain are shown in blue. Post-translationally modified lysine residues found by mass spectrometry are shown in red. B) The arginine residues on Ig heavy chain are shown in blue. Post-translationally modified arginine residues found by mass spectrometry are shown in red.

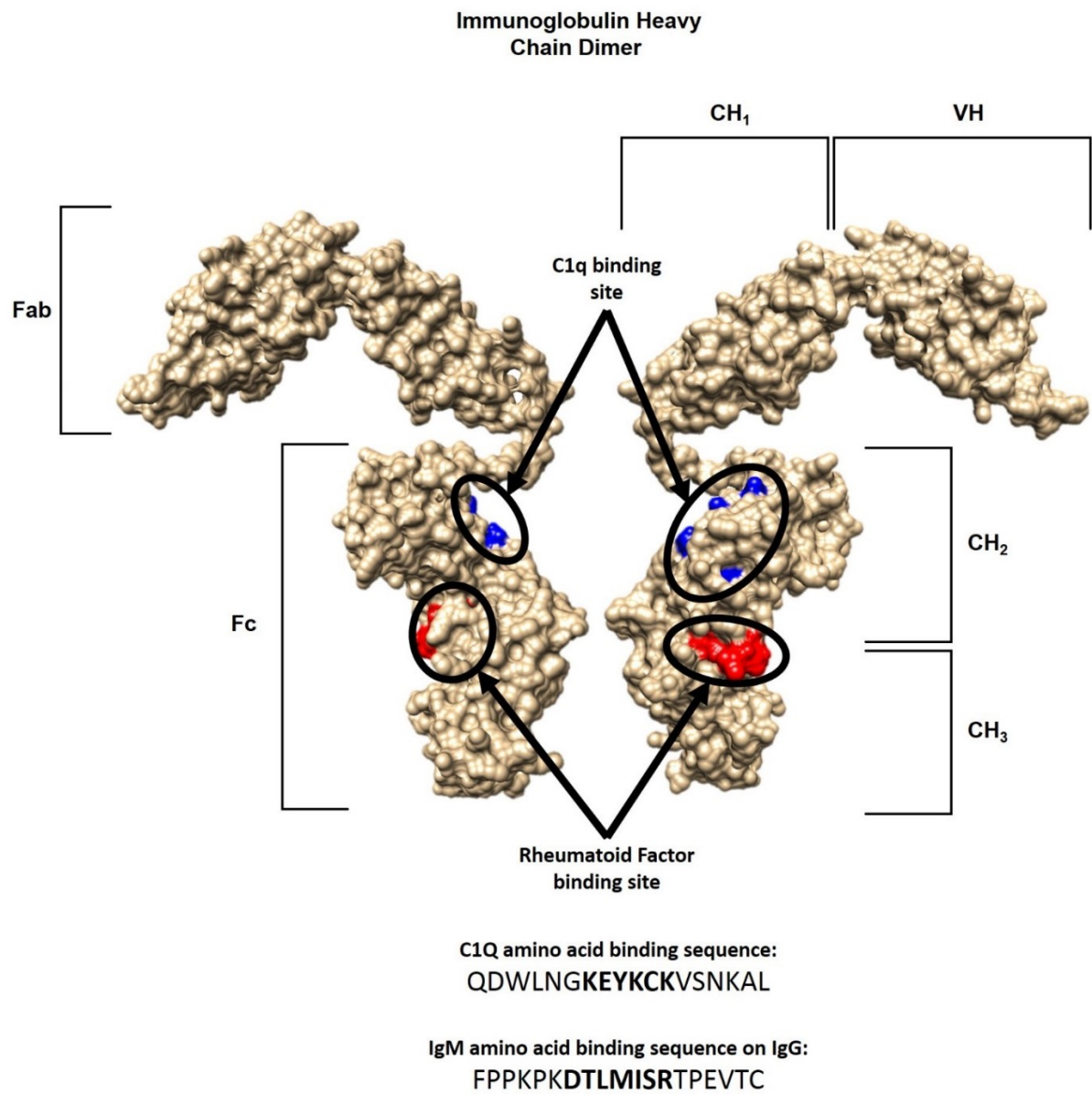


Figure 5-7 Protein model of the heavy chain of immunoglobulins, the sub regions of the molecule, and the binding sites of C1Q and rheumatoid factor.

The C1q binding site is highlighted in blue. The RF binding site is highlighted in red.

Sample ID	Disease Cohort	C1q binding site mod?	RF binding site mod?	Other CH2 region mods?	Other IgG region mods?	Sample ID	Disease Cohort	C1q binding site mod?	RF binding site mod?	Other CH2 region mods?	Other IgG region mods?
1	Br _{sero}	Carb	Carb/Cit	Carb/Cit	Carb	19	HC(S)	Carb	Carb/Cit	Carb	Cit
2	Br _{sero}	No	No	Carb	Carb	20	HC(S)	No	No	Carb	Carb
3	Br _{sero}	No	No	No	Carb/Cit	21	HC(S)	No	No	No	Cit
4	Br _{sero}	No	No	No	Carb	22	HC(S)	Carb	No	Carb	Cit
5	BRRA	No	No	No	No	23	RA	Carb	No	No	Carb/Cit
6	BRRA	Carb	Carb	Carb	Carb	24	RA	Carb	No	No	Carb/Cit
7	BRRA	Carb	Cit	Carb	Carb/Cit	25	RA	Carb	Carb	No	Carb
8	BRRA	No	No	No	Carb	26	RA	Carb	No	No	Carb/Cit
9	RA	No	Carb	Carb	No	27	RA	Carb	No	Carb	No
10	RA	Carb	Cit	Carb/Cit	Cit	28	RA	Carb	No	No	Cit
11	RA	No	No	No	Cit	29	RA	Carb	No	No	Cit
12	RA	No	No	No	No	30	RA	Carb	Carb	No	Cit
13	HC(NS)	Carb	No	Carb	Carb	31	RA	Carb	No	No	Carb/Cit
14	HC(NS)	No	No	No	No	32	RA	Carb	No	No	Cit
15	HC(NS)	Carb	No	Carb	No	33	RA	Carb	Cit	No	Cit
16	HC(NS)	Carb	No	Carb	Carb/Cit	34	RA	Carb	No	No	No
17	HC(NS)	No	No	Carb	Carb	35	RA	Carb	Carb	No	No
18	HC(S)	No	No	No	Carb/Cit	36	RA	Carb	No	No	No

Table 5-4 Summary of the post-translational modifications found in all of the

Each column details if the region in question was found to be carbamylated (carb) or not (no). Carbamylations are found frequently in RA, many samples in the region where IgM b

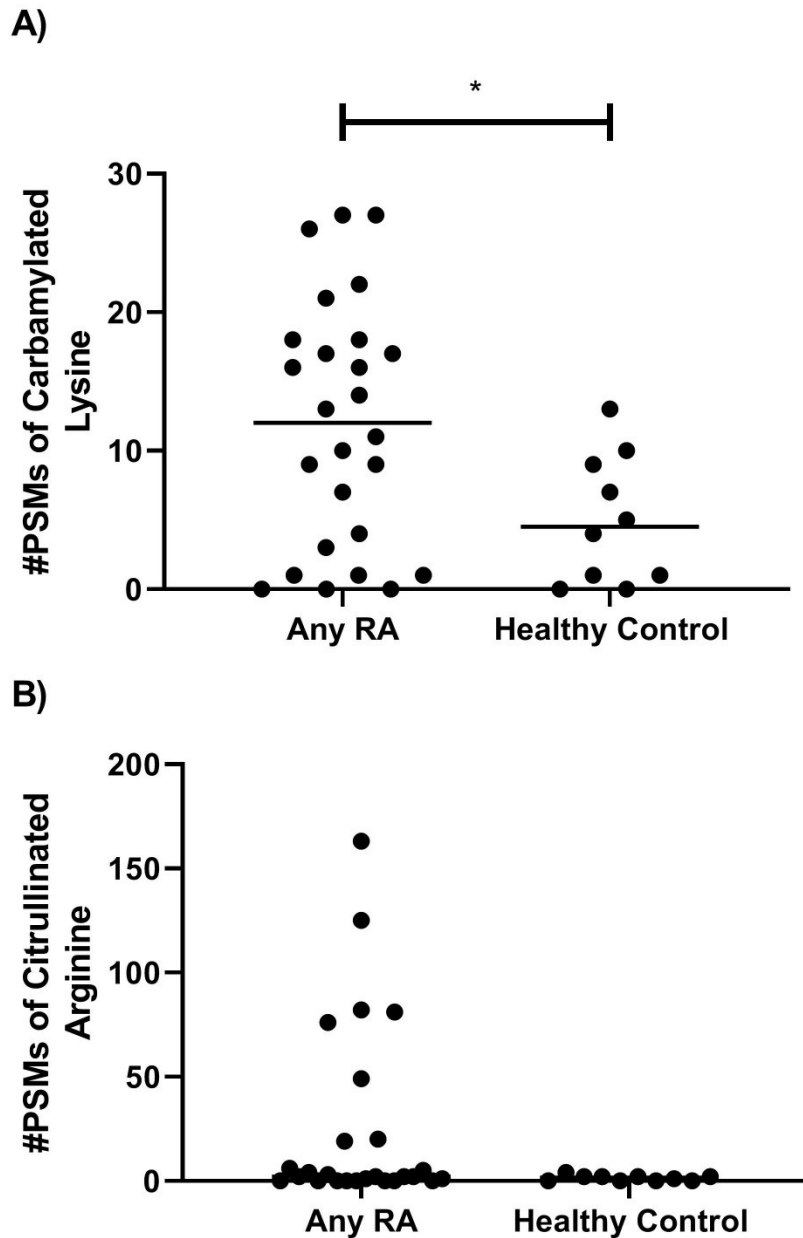


Figure 5-8 Carbamylated lysine residues, but not citrullinated arginine residues in the heavy chain of immunoglobulins are detected in significantly higher quantities in RA serum than in healthy control samples.

The PSM counts for carbamylated lysine residues were totalled for each serum sample tested. Carbamylation was statistically significantly higher in RA_{any} samples compared to healthy control samples. Statistical significance was tested by Mann-Whitney. * = $p < 0.05$

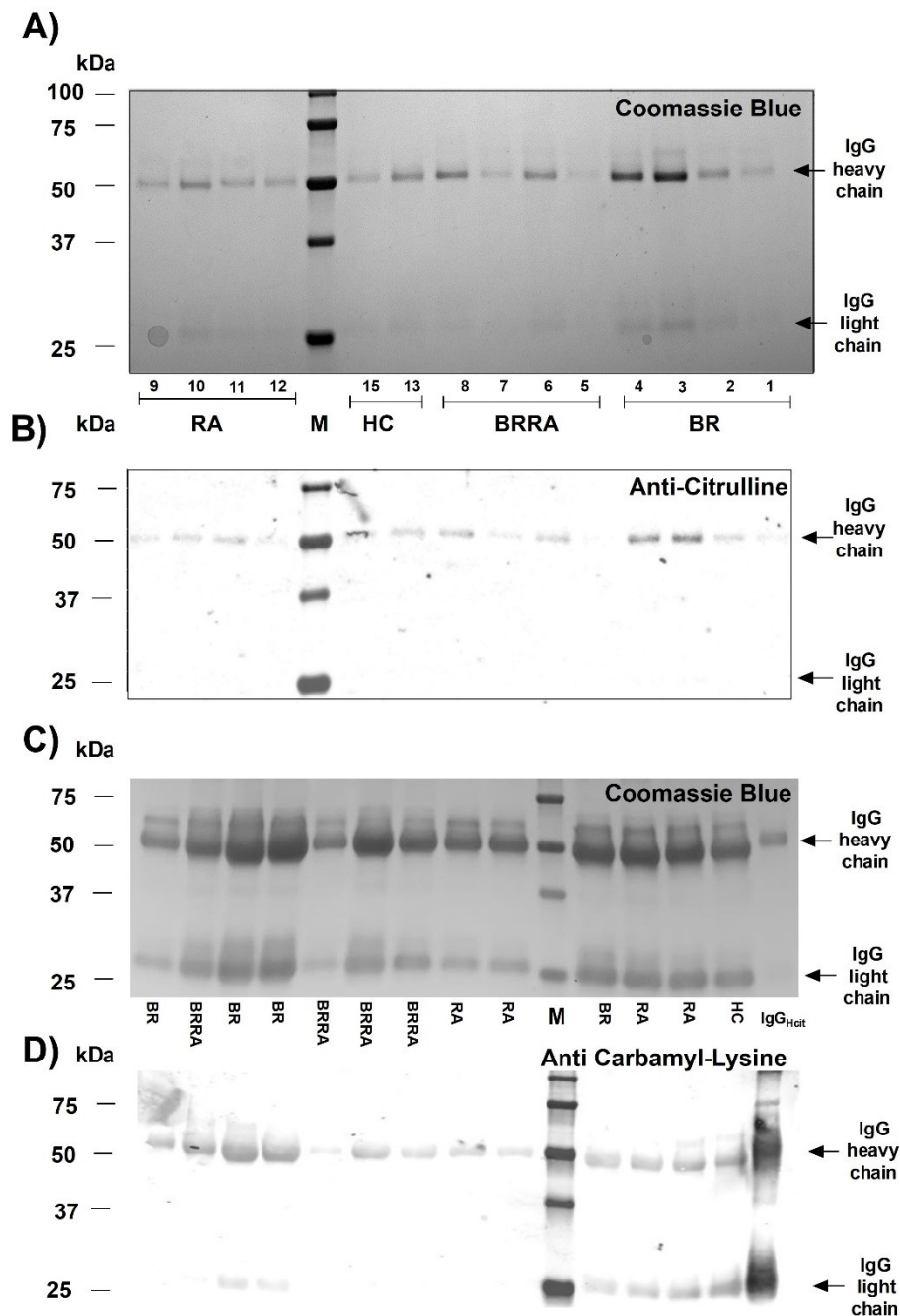


Figure 5-9 SDS-PAGE and immunoblots of purified IgG for citrullination and carbamylation.

A) Representative Coomassie-blue-stained isolated total IgG from patients and control sera used to blot for citrulline. B) Immunoblot of IgGs probed with anti-citrulline. C) Representative Coomassie-blue-stained isolated total IgG from patients and control sera used to blot for carbamylation. D) Immunoblot of IgGs probed with anti-carbamyl-lysine. For C) and D), individual sample cohort identifiers are shown.

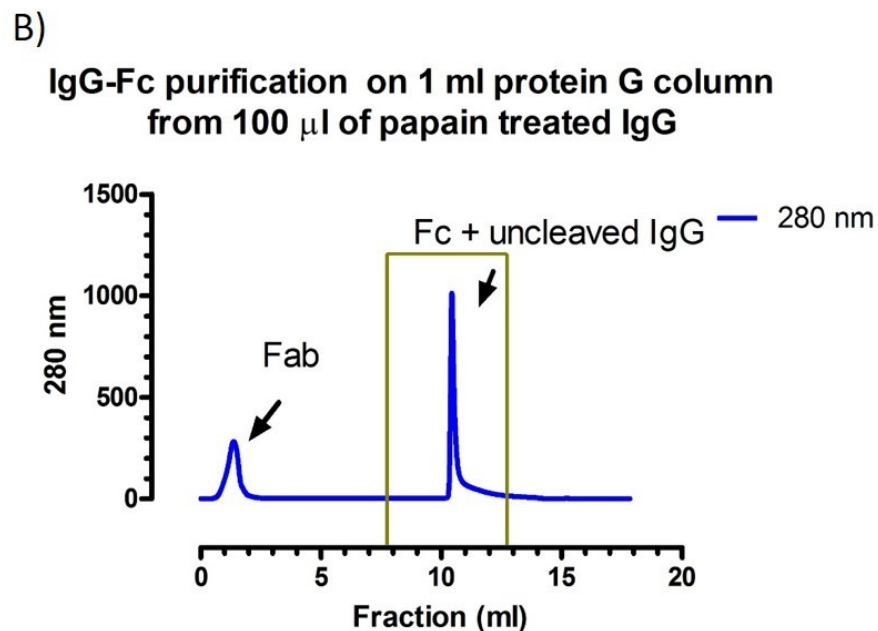
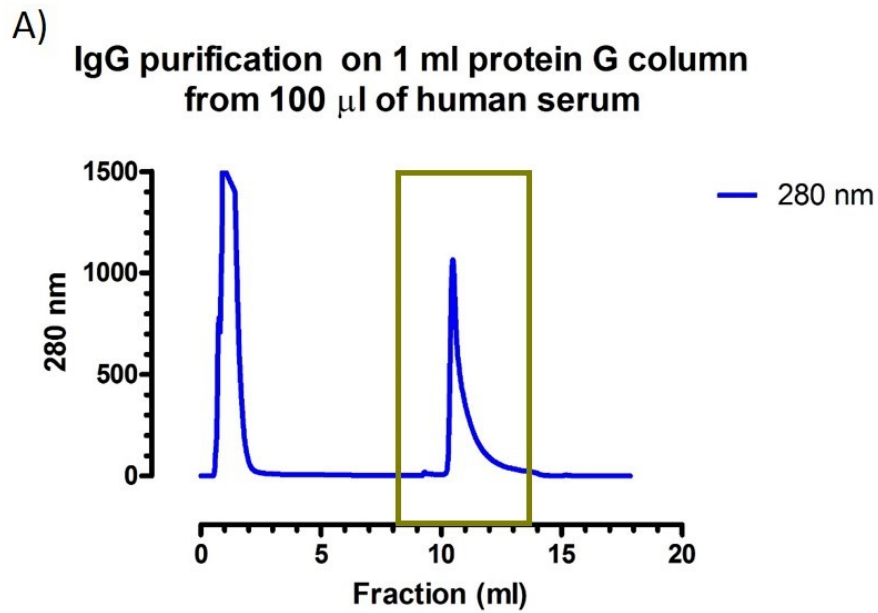


Figure 5-10 Fast protein liquid chromatography confirmation of IgG and Fc fragment purification.

A) IgG purification from commercially available serum. B) Papain treated IgG samples that were repurified via FPLC. The green boxes represent the elution window through which IgG or Fc fragment bound to the column was eluted to reveal pure protein. A_{280} represents an estimate of protein concentration.

To test this hypothesis, Fc fragments were generated and the serum antibody response against them was measured. To generate the Fc fragments, IgG from commercially available serum was purified through FPLC (**Figure 5.10A**).

Once purified, papain was used to digest the bonds between the Fc region and the Fab region of IgG. Papain was chosen to preserve the disulphide bonds between each heavy chain, in the Fc fragment, to mimic the biological Fc component of IgG as closely as possible. Upon papain treatment, samples were run once again on the FPLC, using as protein G column for purification.

During repurification, the cleaved Fab region of IgG can be seen in **Figure 5.10B** as the small peak to the left of the elution window. The pure Fc fragment, or uncleaved IgG, then eluted off of the protein G column. With eluted protein consisting of either cleaved Fc fragment, or uncleaved whole IgG, SDS-PAGE was performed to confirm if full cleavage had taken place. The post-papain IgG elution lacked the presence of the heavy chain typically found in the 50-60kDa region in IgG. As samples were reduced using DTT, the majority of protein in the Fc isolate was in the 22-28kDa region, indicating that papain cleavage had taken place and therefore Fc fragment had been successfully isolated (**Figure 5.11**). After Fc fragment isolation, the protein was citrullinated and carbamylated to mimic the protein species observed by mass spectrometry. As the process of citrullination is enzymatic (PAD-driven), and carbamylation is chemical (potassium cyanate driven), the Fc fragment was first citrullinated and then carbamylated to avoid any potential changes to the Fc fragment that may make the enzyme:protein reaction unsuccessful.

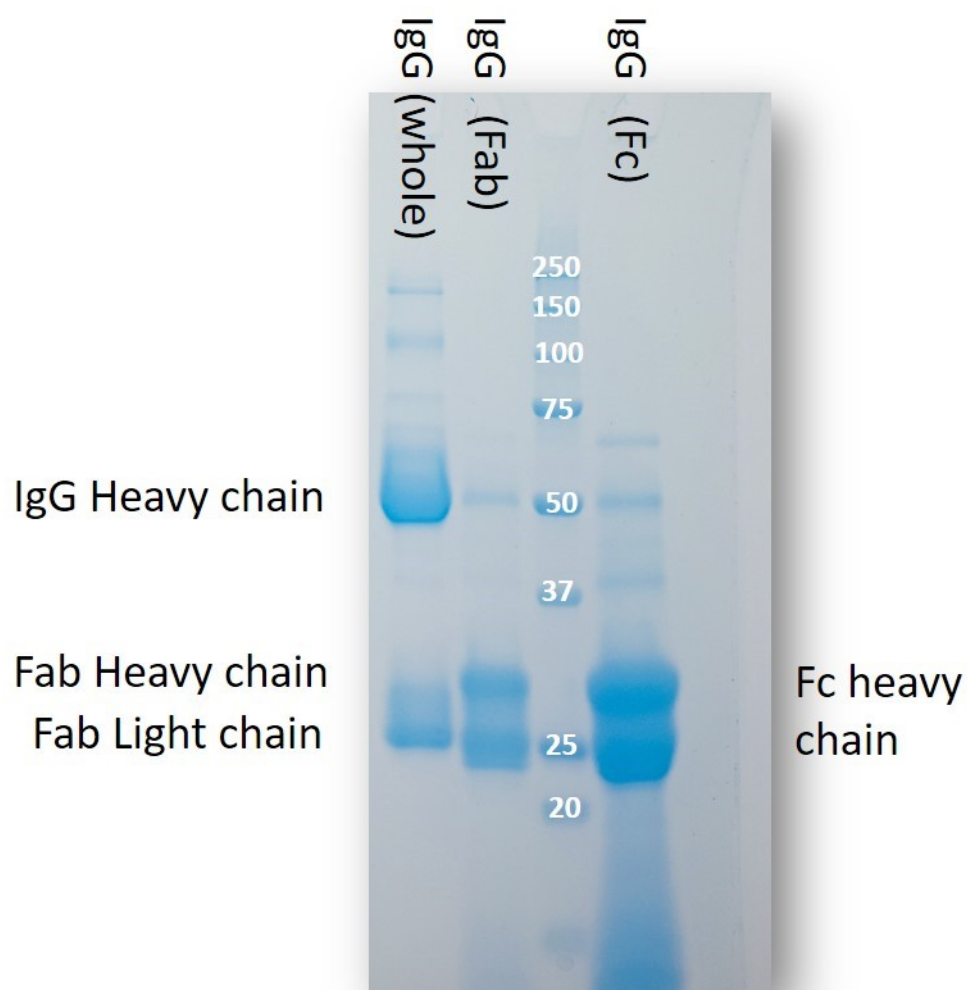


Figure 5-11 Fc fragment cleavage was confirmed via SDS-PAGE.

Whole IgG isolates, runoff from FPLC post-papain cleavage, and eluted IgG from FPLC post-cleavage was loaded onto 8-16% Tris/Glycine/SDS gels and stained with Coomassie blue.

After modification of the Fc fragment was analysed via mass spectrometry to confirm that citrullination and carbamylation had taken place, and determine if PAD was capable of enzymatically modifying this protein *in vitro*.

Mass spectrometry revealed that carbamylation had occurred on most lysine residues, though citrullination had occurred at lower levels. Having successfully modified the Fc fragment, the serum antibody response was measured in the samples collected earlier.

5.5 Assessment of serum antibody response to unmodified and citrullinated and carbamylated Fc fragment

As rheumatoid factor is an immune complex composed of IgM or IgA bound to IgG (**Section 1.4.1.3**), the antibody binding to the Fc fragments was assessed (as outlined in the template shown in **Methods Table 2.2**).

A 1:50 dilution of serum from BR, BRRA, RA, and HC serum samples was first used to ascertain if the antibody responses differ between different disease cohorts and healthy samples.

In total, over 400 serum samples were screened consisting of all the RA (n = 266) and BRRA (n = 50) and HC samples (n = 71) , in addition to select BR samples (n = 32) were tested. The results from the ELISA analysis are shown below.

All of the absorbance values for the four parameters tested can be seen in **Figure 5.12A-D**. In the unmodified Fc fragment response (**Figure 5.12A**), the RA and BRRA IgM response is significantly higher than the HC ($p < 0.0001$ for both) and BR ($p = 0.0443$ and $p = 0.0141$, respectively).

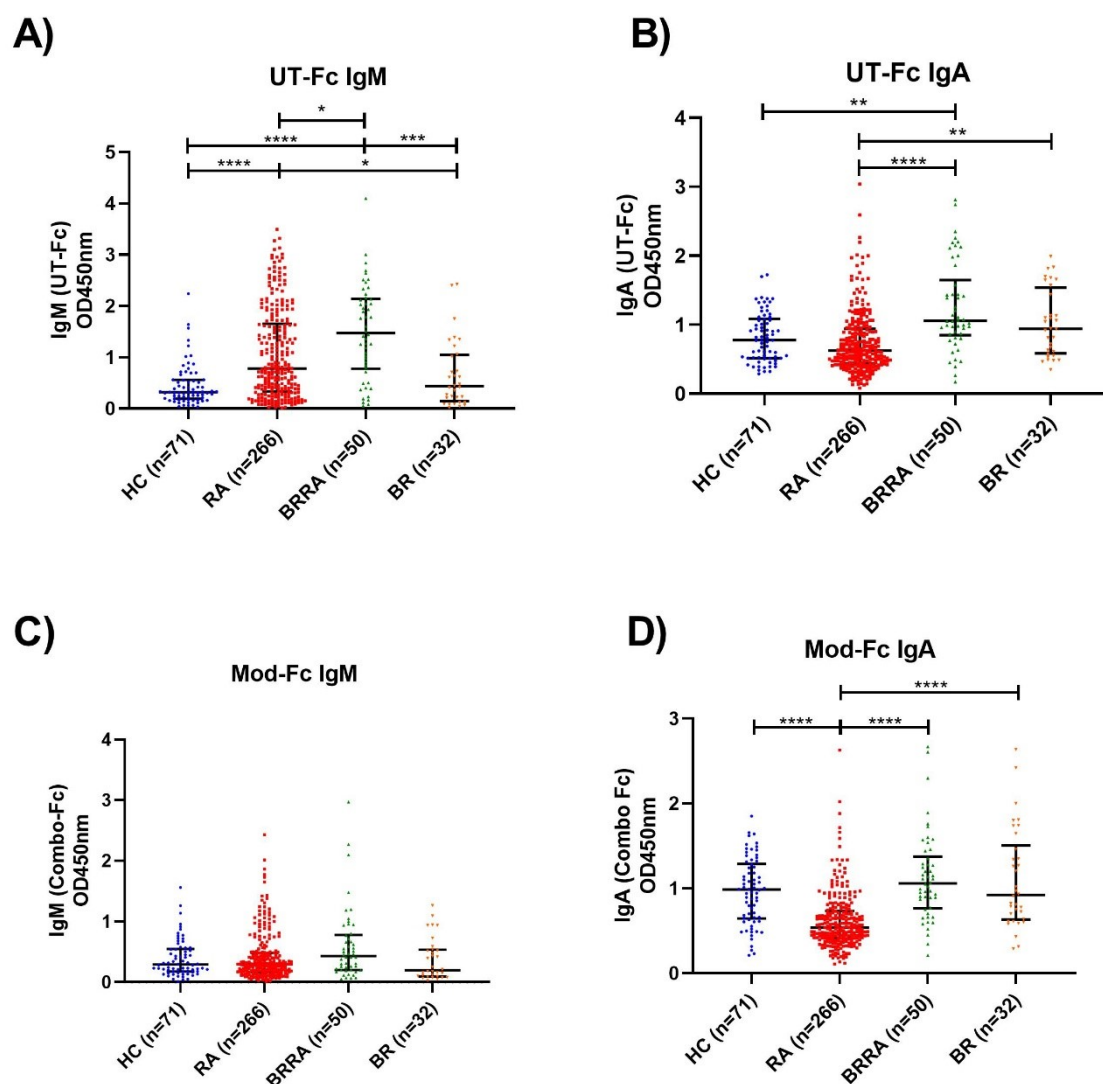


Figure 5-12. The absorbance values for the serum IgM and IgA response to unmodified and double-modified Fc fragment.

A) Serum IgM response to unmodified Fc fragment. B) Serum IgA response to unmodified Fc fragment. (C) Serum IgM response to citrullinated and carbamylated Fc fragment. (D) Serum IgA response to citrullinated and carbamylated Fc fragment. Statistical significance was analysed using a Kruskal-Wallis test with Dunn's comparison post-test. * $p < 0.05$ ** $p < 0.005$ *** $p < 0.001$ **** $p < 0.0001$. Bars are displayed as median with interquartile range. HC = Healthy Control, RA = rheumatoid arthritis, BRRA = bronchiectasis with rheumatoid arthritis, BR = bronchiectasis only.

This difference is completely negated in the serum IgM response to citrullinated and carbamylated Fc fragment, where the response seen is not significantly different across all cohorts tested (**Figure 5.12C**).

In the context of the IgA response, the BRRA cohort demonstrated significantly higher titres of IgA binding to the unmodified Fc fragment compared to HC ($p = 0.0029$) and RA ($p < 0.0001$) serum samples (**Figure 5.12B**). The BR cohort also displayed significantly higher binding than the RA cohort ($p = 0.0037$). Perhaps the most interesting finding from the inter-cohort comparisons however, is that the IgA response to citrullinated and carbamylated Fc fragment is statistically significantly lower ($p < 0.0001$) in the RA cohort compared to all other cohorts tested (**Figure 5.12D**). No difference in OD450_{nm} was seen between the HC, BRRA and BR cohort responses.

Further examination of the changes in IgM and IgA response between unmodified and cit/carb Fc fragment were performed. Both the RA and BRRA cohorts had a significantly lower ($p < 0.0001$ for both) IgM response to mod-Fc than unmodified Fc fragment (**Figure 5.14**). The IgA response to mod-Fc in HC samples was significantly higher than unmodified Fc ($p = 0.0223$), whilst the RA response was significantly lower ($p = 0.0016$).

Finally, the OD450_{nm} values were compared against anti-CCP, RF and DAS28 scores of the same samples. The rationale for this was to see if anti-citrullinated protein antibody titres (through anti-CCP measurement) influenced the response to Fc fragment that had been citrullinated. Similarly, as the IgM binding site of IgG in RF was found to be post-translationally modified, that the RF titre may somewhat influence findings also.

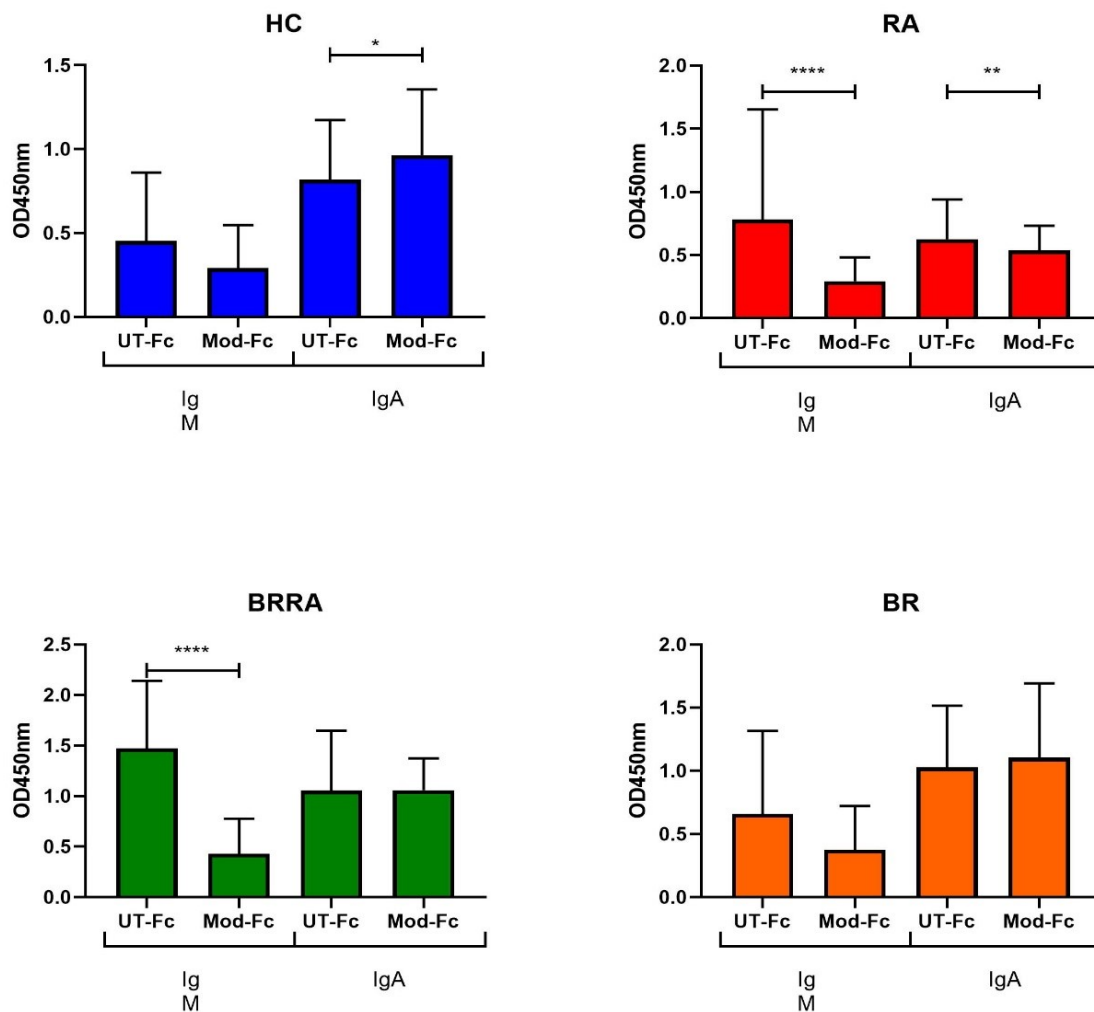


Figure 5-13 Histograms displaying the change in serum antibody binding between unmodified and citrullinated and carbamylated (mod) Fc fragment.

Each cohort response is shown. In RA and BRRA cohorts, the serum IgM response to mod-Fc is significantly lower than the unmodified response. In the HC cohort, the IgA response to mod-Fc is significantly higher than the response to the unmodified Fc fragment. In the RA cohort, the serum IgA response to mod-Fc is significantly lower than the unmodified fragment. Statistical significance was tested by Mann-Witney test.

* $p < 0.05$, ** $p < 0.005$, **** $p < 0.0001$. Values are displayed as median $OD_{450nm} \pm$ interquartile range. HC = Healthy Control, RA = rheumatoid arthritis, BRRA = bronchiectasis with rheumatoid arthritis, BR = bronchiectasis only.

Paradoxically, for anti-CCP, RF and DAS28, the strongest correlations for both the subjects IgM and IgA binding to IgG were found in the unmodified Fc fragment groups as opposed to the citrullinated and carbamylated fragments of IgG (**Table 5.4**).

The same pattern is seen in the BRRA cohort for the IgM response also, with these parameters also correlating more strongly in the UT-Fc response compared to the mod-Fc response. Interestingly, for the IgA response however, mod-Fc correlates more positively than UT-Fc for anti-CCP, RF and DAS28 (**Table 5.4**).

Antibody response	Cohort	Anti-CCP titre (U/ml)	Rheumatoid Factor (U/ml)	DAS28-CRP
IgM:UT-Fc	RA	0.2124***	0.5653****	0.1577*
	BRRA	0.4873***	0.7578****	0.1899
IgA:UT-Fc	RA	0.192**	0.3335****	0.133*
	BRRA	0.3917**	0.503***	0.07018
IgM:Combo-Fc	RA	0.198**	0.461****	0.1338*
	BRRA	0.3896**	0.6661****	0.2622
IgA:Combo-Fc	RA	0.08647	0.17**	0.153*
	BRRA	0.4204**	0.6074****	0.1648

Table 5-5 Spearman's rank correlation coefficient for OD450_{nm} versus clinical serological scores for rheumatoid arthritis patients with and without bronchiectasis.

In each cell is the r value calculated by spearman's rank correlation coefficient, and the level of statistical significance measured. * p<0.05, ** p<0.005, *** p<0.001, **** p<0.0001

5.6 Discussion

In this chapter, it has been shown that in RA patients with and without bronchiectasis that the heavy chain of IgG, and alpha-1 antitrypsin display high levels of citrullination and carbamylation compared to healthy people.

5.6.1 Implications for citrullination and carbamylation of alpha-1 antitrypsin

Alpha-1 antitrypsin has long been known to be associated with RA pathogenesis (379, 380). Alpha-1 antitrypsin is a serine protease of which the primary target is neutrophil elastase, the antimicrobial protein released by neutrophils during bouts of inflammation (381). Additional functions of this protein focus on a modulatory role, with A1AT modulating IL-8 and TNF α levels (382). Deficiency of this enzyme, most commonly through genetic defects (A1ATD), is associated with COPD, bronchiectasis, and autoimmunity in general (383-387). The discovery of citrullination and carbamylation of A1AT in these samples is of great importance, as PAD2 and PAD4 release is increased in A1ATD patients, suggesting that A1AT may have some role in ACPA generation (388). The authors of this study also found increased quantities of citrullinated proteins and PAD4 present in the BAL fluid of A1ATD patients. Deficiency of A1AT has also been detected in a subset of RA patients, with heterozygosity for the deficiency gene leading to increased ACPA titres in RA patients (382). Furthermore, A1AT therapy through intravenous infusions, or gene therapy improves the symptoms of mice experimentally given RA (380). Carbamylated A1AT is a target of anti-CarPs in RA patients, which exist in higher levels compared to healthy controls (389). These data confirm the findings of Verheul *et al*, whilst adding to these through discovery of citrullinated arginine residues on this protein also. As previously discussed, citrullination and

carbamylation, though different PTMs, share the same result in that protein function or structure may be changed due to neutralisation of the amino acid charge on the lysine or arginine residue. High levels of citrullination of Arg220 of A1AT in RA patients significantly correlated with ACPA titres in the samples tested.

This may suggest that in RA, this protein is wholly post-translationally modified, potentially resulting in loss of function. The presence of anti-CarPs to this protein also pose the question of whether or not ACPAs directed against *citrullinated* A1AT exist also. Nevertheless, the implications of these PTMs present an *in vivo* opportunity for NE to remain active for longer periods of time, potentially contributing to damage of host tissue during bouts of inflammation. In BR patients, NE activity correlates with the rate of decline in lung function and infections (390, 391). This may mean that in BRRRA patients, A1AT modification could be one of the initiating mechanisms through which autoantibody formation occurs.

5.6.2 Implications for citrullination and carbamylation of the heavy chain of IgG

Carbamylation of IgG has been identified *in vivo* previously by Koro and colleagues (392). This work adds to these findings through the additional presence of citrullination of IgG in RA patients. Of particular note is the carbamylation and citrullination observed at or near the binding site of the C1q complement protein, or the IgM binding site in rheumatoid factor (234, 377). As stated in the introduction, the mechanisms through which RF forms is however unknown (**Section 1.4.1.3**).

Although the interaction between IgM and IgG is well known as RF, the more frequently observed modifications to the C1q-binding region in this study are

more interesting. General carbamylation of IgG substantially decreases C1q binding and was found by Koro *et al* to decrease the incidence of complement-mediated cytotoxicity (392). By inhibiting complement activation through modification to this region, this prevents further activation of the complement cascade. The complement cascade is integral to removal of immune complexes, and decreased clearance of immune complexes is associated with the pathogenesis of not just RA, but systemic lupus erythematosus (393, 394). Adversely, complement protein complexes are elevated in the synovial fluid of RA patients, with C1q-C4 complexes correlating with disease activity scores, thus calling into question the functional consequences of carbamylation of the C1q binding site (395).

On the one hand, Koro *et al* used IgG that had been chemically modified *in vitro*, and tested the complement protein response to this modification, potentially resulting in more exaggerated levels of the modification taking place, and reporting drastically lower interaction between C1q and IgG. This could mean that *overmodification* of the protein results in reduced binding capacity from structural changes to the protein closing off the binding site of C1q. Citrullination and carbamylation of certain proteins results in greater binding affinity to certain targets, suggesting that *in vivo* levels of these modifications may enhance the binding affinity, leading to overactivation in RA (396-398). Carbamylation also increases the hydrogen bonding capacity of the protein in question, further strengthening the idea this modification enhances binding affinity, rather than inhibiting it (399). This is supported by a wide body of evidence showing that immune complexes activate the complement system rather avidly (400-404).

The presence of carbamylation of this site so frequently in RA samples used in the mass spectrometry analysis cements the notion that modification of this site plays some sort of role in RA pathology, though the effect on complement activation is currently unknown. It could be that the physiological or pathological levels of carbamylation that occurs in the IgG molecule (as shown by these data) enhances biological activity. However, *in vitro* chemical modification may produce non-physiological levels of carbamylation which inhibit C1q binding. This would be an intriguing avenue for further experimentation.

Although Koro *et al* focused on the ability of C1q to bind IgG, and the associated complications, the modifications seen in the RF binding site propose the first possible mechanism through which the RF autoantibody may develop. Carbamylation of the CH2 region of IgG has been reported previously *in vivo*, with the PPKPK motif close to the RF binding site being also detected by Koro *et al* through *in vitro* carbamylation (392).

The assays that measure RF titres in patient serum typically use recombinant IgG, or Fc fragments to assess the presence of autoantibodies (405). As PTMs were found in the Fc region in RA patients, a novel approach was used to modify the Fc component of IgG to either confirm that citrullination/carbamylation is the driving mechanism for RF formation, or potentially devise a more sensitive or specific diagnostic assay for RA patients.

5.6.3 The effect of citrullination and carbamylation on the serum IgM and IgA response to Fc fragment

The results from the ELISA paint two very different pictures of serological outcomes, based on whether or not the RA patients were also suffering from

bronchiectasis. Unsurprisingly, for the IgM response, the untreated Fc fragment correlated the most strongly for both RA and BRRA patients. This refers to the experimental principle behind the RF clinical assay itself, in that this was effectively the most similar experimental design to the clinically-used assay.

The diminished response in all cohorts to the mod-modified Fc fragment is unexpected, as the RA and BRRA patients had demonstrated high levels of carbamylation to the CH2 region of their respective IgG heavy chains, and therefore should have a greater antibody titre to this modified isoform of the Fc fragment. An additional hypothesis to explain the results could be other confounding factors not accounted for in the experimental design. Serum components such as complement proteins may play an influence in the antibody binding in this assay. Furthermore, given the complex system of antibody-antibody-antibody interactions with this assay, the secondary antibodies used in the assay could also highlight potential explanations for the results obtained.

Perhaps the hypothesis put forward for the C1q-IgG interaction seen in the study by Koro *et al* is the same for the results observed from this ELISA. The over-carbamylation of this protein may have prohibited anti-carbamylated Fc IgM antibodies from binding to their respective epitopes, leading to a reduced response. As titres were low across all 400 samples tested compared to UT-Fc, it may also be that antibodies to these modified epitopes are actually less abundant, and that the autoantibodies in the RA/BRRA cohorts were always directed against non-modified residues in IgG, perhaps indicative of a more advanced stage of RA, in which IgG itself, in an unmodified form is the target of autoantibody production.

A different perspective is observed in the context of the IgA response, however. Firstly, IgA titres are elevated in RA patients and there is roughly 50% more IgM-RF than IgA-RF (406-408). The fact that the serum IgA response to mod-Fc correlated more strongly with anti-CCP, RF and DAS-28 than UT-Fc is intriguing. The reasons for this are perplexing, given that the proposed reasons for reduced IgM binding in the mod-Fc should logically be reflected in the IgA binding also. Therefore, it may be differences in the IgA molecule itself that may account for the binding capacity of the protein. IgA in serum typically exists as a monomer, in addition to having a smaller CH3 region, similarly to IgM (409). As IgM is a pentameric molecule, it may be that this monomeric immunoglobulin is able to physically bind the protein in ways through which IgM may not. If this were to be wholly true however, then the RA only cohort would also demonstrate the same outcome, which it does not. Then perhaps, it is a unique feature to RA patients with underlying BR, that give rise to this unique result. The significance of these findings has yet to be elucidated.

Immunoglobulin A titres are increased in BR patients. Further to this, IgA is synthesised in the body at a much higher rate than IgM at mucosal surfaces (409). Bouts of pulmonary inflammation are all too common in BR patients, as recurrent infections are one of the most frequent symptoms of the disease (258). This chronic inflammation, coupled with the high synthesis of IgA, may create a unique microenvironment specific to BRRA patients, through which PTM of IgA readily occurs. The presence of IgA-RF predates the onset of RA by roughly 10-15 years, suggesting that mucosal dysfunction may be the driving factor in autoantibody development (205, 206, 217). It is plausible then to hypothesise that in BR patients, IgA titres are elevated, immune cell overactivation occurs, and thus more likely to generate autoantibodies to

citrullinated and carbamylated proteins, in this case IgG. These IgA-IgG_{FC} antibodies then circulate in the body, kept highly present through chronic inflammation, thus explaining the ELISA results obtained.

The problem then, is how the other cohort IgA responses fit into this hypothesis. In the RA cohort, the serum IgA response to mod-Fc fragment is significantly lower than the unmodified variant, and the HC samples result in a greater IgA-Fc response in both UT-Fc and Mod-Fc. The results seen in the RA cohort contradict the literature somewhat, as IgA-RF is significantly elevated in RA patients compared to healthy controls (205, 410-415). The only unique factor to this cohort is that 30-40% of the samples are RF-negative, which may account for the low ODs seen in the ELISA. It would be of great importance to measure the differences in RF subtypes (IgM-RF, IgA-RF and IgG-RF) in these samples in order to see if the IgA-RF response reflects the results observed here in the IgA:UT-Fc response.

5.7 Conclusion

In this chapter, it has been shown that in a group of 24 RA samples with and without BR, that A1AT and the heavy chain of IgG are both citrullinated and carbamylated to varying degrees, though in greater quantity than those seen in healthy control samples. Of note is that in RA patients, these modifications were localised to two specific regions: the C1q binding site, and the IgM binding site found in RF.

In light of this finding, Fc fragments of IgG were generated that were either untreated, or citrullinated and carbamylated, and measured the serum IgA and IgM to these fragments by ELISA. After testing over 400 samples, it was found that the IgM response to unmodified Fc fragment was stronger than the mod-Fc

fragment in RA and BRRA patients, and that RF, anti-CCP and DAS-28 correlated strongly with the IgM:UT-Fc response. The IgA response in RA patients was lower than healthy control samples, for unknown reasons. The IgA response in BRRA patients was stronger for the mod-Fc fragment than the untreated Fc fragment, potentially explained by the influence of bronchiectasis underlying the RA disease.

6 Overall discussion and future work

6.1 Summary of results

The experiments of this thesis centre on a core rationale of investigating potential pathways through which immune tolerance can be broken in RA. This project consisted of three questions: one prospective, and two retrospective.

The rationale behind these questions can be seen in **Figure 6.1**.

The first question of “does cadmium exert any changes in neutrophils” is nuanced. On the one hand, it was found that cadmium did not alter the function of three vital neutrophil processes: the respiratory burst, phagocytosis and NETosis. However, it was demonstrated that cadmium exposure in neutrophils, results in increases in cytosolic calcium levels, which may affect secondary messaging systems inside the cell. Furthermore, this work shows that cadmium may, however, prime both the respiratory burst and NET response for subsequent stimulation by PMA; this may be attributable to cadmium altering protein kinase c function through displacement of the zinc ion on the protein, resulting in greater activation (348).

This now paints a picture of cadmium not directly causing neutrophil function but potentially acting as a priming agent through which subsequent stimuli result in an exaggerated response. This exaggerated response may result in greater levels of extracellular carbamylation in activated neutrophils, as discovered by Nakabo *et al* (281). This hypothesis is supported by evidence that cigarette smoke exposure in rabbits results in carbamylation of vimentin, and subsequently autoantibodies to this carbamylated variant (233). Cigarette smoke contains various toxic compounds, though it is the largest source of cadmium for the general population. Further to this, it was demonstrated that cadmium plays a part in calcium release and PKC activation, which are both required to cause neutrophil degranulation (416).

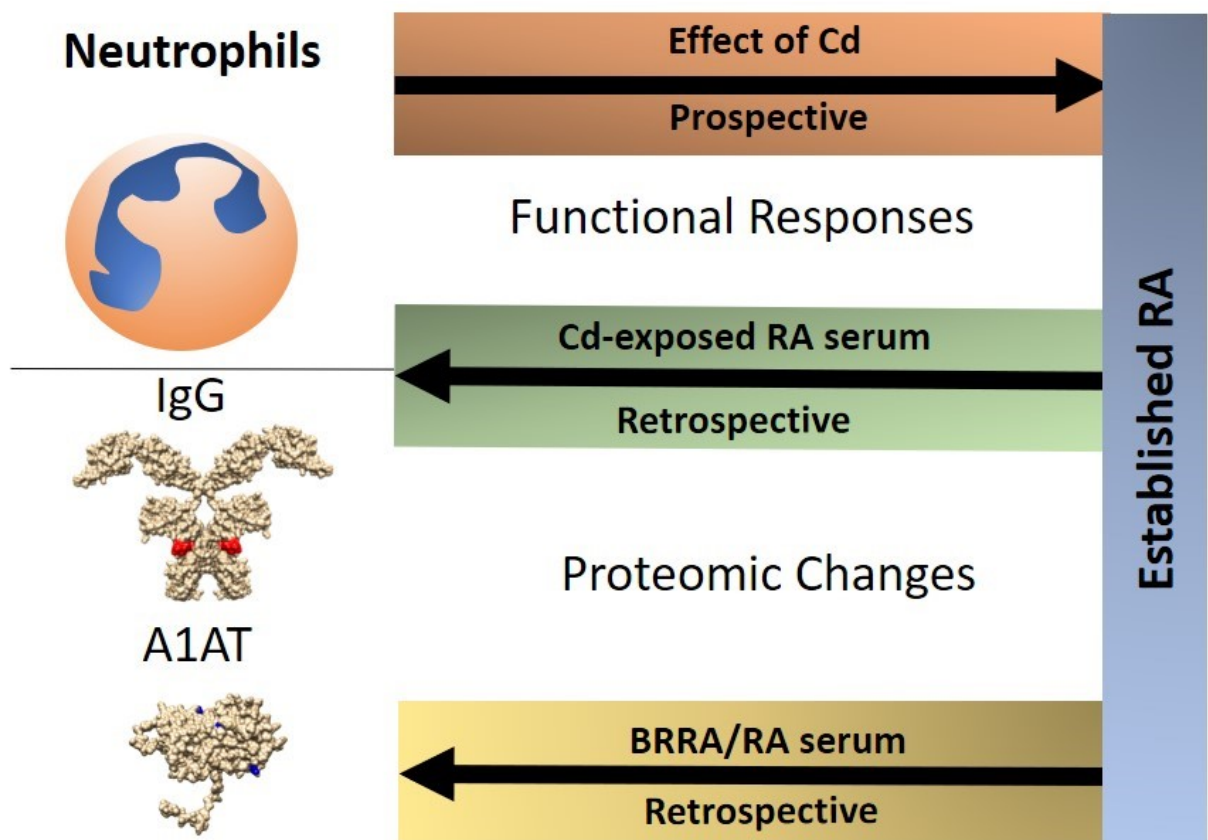


Figure 6-1 Summary of the rationale behind the experiments undertaken during this thesis.

In chapter 3, the effect of cadmium on neutrophil functions was assessed. In chapter 4, the neutrophil response to RA serum from select patients with varying degrees of Cd exposure was investigated. In chapter 5, the proteomic changes in RA patient serum samples was analysed against healthy control serum, to see if any stark contrasts were observed. The neutrophil function response in chapter 4 serves as the link between chapters 3 and 5, tying the link between neutrophil functional responses, and RA serum proteomic changes together.

Therefore, it is proposed that one mechanism through which cigarette smoke contributes to breakdown of immune tolerance, is through Cd-induced exaggeration of PKC activation, resulting in greater levels of MPO release. Rats exposed to Cd demonstrated increased levels of intracellular MPO levels, lending further strength to this argument (308).

To see if these findings possibly persisted in established RA patients, previous neutrophil functional assay work was replicated with RA serum, with samples consisting of various levels of urinary cadmium, in addition to differing serological scores between each sample. The lack of functional responses across all assays used are confusing, given that RA serum is known to stimulate healthy neutrophils in certain processes, such as the respiratory burst (368). Further to this, the response in one serum sample with the highest level of urinary cadmium, and relatively high RF and anti-CCP and DAS28 scores may suggest that any influence that cadmium may have on immune system functioning in RA patients appears to have long since ceased, as no stimulatory effect was seen in this serum sample. Obviously, this is merely the finding of one unique patient sample, and so more samples from patients that share these characteristic traits would lend more strength to these data.

It was previously discussed reasons for why these experiments did not replicate prior findings, but the low number of serum samples used overall (n=8) may be insufficient to state any conclusions found from the respiratory burst or calcium flux assays. Even more confusing, is the large quantity of RA patient serum used (n=44) in the NETosis induction experiments, once again confounding current literature, with RA serum inducing less extracellular DNA release than healthy serum samples. Experimental differences may account for this, but

taken altogether, it would suggest that cadmium does not attenuate neutrophil function adversely in RA patients with a long-standing history of Cd exposure.

This then means that in a biological setting, the only role that cadmium may play in any aspect of immune system functioning, is potentially in the initial stages of RA development. Acute doses of Cd exposure in the lungs are the most likely scenario through which this dysfunction occurs, typically through smoking. The lungs are more likely than the gut to be the site of this, as cadmium nanoparticles citrullinate proteins in lung cells, with cytokeratins being the most common target (417). Citrullinated cytokeratins are also present in the joint of RA patients, further highlighting another mechanism through which cadmium may be implicated in RA pathogenesis (418).

And so the other side of lung-induced RA development is highlighted through these experiments into analysing a portion of the proteome in BRRA/RA patients. The significance of this cohort of patients was established by this group and has built on this strong foundation with additional important findings. Firstly, and most notably, the findings of Koro *et al* have been confirmed regarding carbamylation of IgG (392). Whereas they modified IgG *in vitro*, It has shown for the first time, that these same modifications can occur *in vivo* in RA patients. Even more notably, is the discovery of these same modifications in the location that IgM binds IgG in RF. The significance of this finding may provide potential insights into how this autoantibody immune complex is formed.

This finding was further explored through isolation of IgG and generating pure Fc fragments. Following this, Fc isolates were citrullinated and carbamylated, to attempt to somewhat replicate an isoform of IgG that had been found through mass spectrometry. The hope was that RA patients would have IgM or IgA

antibodies that recognised the modified Fc fragment more avidly than unmodified Fc fragment. Unfortunately, the IgM response in RA and BARRA patients was greater towards the unmodified Fc fragment. The IgA response to UT-Fc and mod-Fc in RA patients was also markedly lower than all of cohorts tested. The IgA response in BARRA patients was similar between the two variants of Fc fragment, though mod-Fc absorbance values correlated more positively with existing serological measurements. The reasons for the diminished response to the mod-Fc fragment could possibly be due to overmodification of the protein in question, making it an imperfect means to assess autoantibody response to carbamylated and citrullinated IgG.

6.2 Future work

An obvious flaw with some of these experiments in this thesis involved the use of the HL60 cell line in certain assays. If it were possible, these experiments would be repeated with primary neutrophils to confirm the initial findings. Additionally, some preliminary experiments were performed in duplicate as opposed to the accepted standard of at least in triplicate. Providing more time was available, the quantity of repeats in some of these experiments would be increased in order to strengthen the data.

The findings of this project provide potential insights into the long-neglected role of cadmium in neutrophil function. The two findings that warrant further investigation are the potential relationship between cadmium exposure and PKC function, and the increase in intracellular calcium levels upon neutrophil treatment with cadmium. These results are obviously very generic in terms of the downstream effects that could occur as a result of these activation pathways. Other functional assays on the effect of cadmium are yet to be investigated, such as neutrophil migration or neutrophil adhesion which may

provide further answers to the question of whether cadmium negatively influences neutrophil biology.

Protein kinase C is crucial to several downstream processes, most notably NADPH oxidase activation in neutrophils. This in turn is critical in activating the neutrophil response, with degranulation, ROS production and NETosis all arising from this pathway. The most important thing to consider about this activation pathway is to attempt to emulate these findings using lipopolysaccharide, not PMA, to best reproduce an infection-based model that would occur in the pulmonary environment. As PMA is a phorbol ester that does not have any biological analogues, it is essential to see if neutrophil responses through lipopolysaccharide-induced activation can be influenced by cadmium in the same manner.

The reasons for not using LPS as a stimulatory compound in this thesis arise from lack of NET induction during the efforts to generate neutrophil extracellular traps despite multiple steps to troubleshoot the issue. Future work would test LPS with a variety of second stimuli such as adenosine triphosphate in order to confirm the general findings in the literature regarding the role of LPS in many neutrophil processes.

Elevations in calcium signalling pose the most open-ended question of what this does to the intracellular signalling. The effect of cadmium on PAD activity in neutrophils is crucial to investigate. With evidence emerging of cadmium-induced citrullination in certain cell types, it could be that cadmium induced PAD activation is possible (417). Myeloperoxidase levels are higher in Cd-exposed mice, though myeloperoxidase activity has yet to be examined. Answering the

question of whether cadmium activates MPO and PAD would then be able to state if cadmium contributes to RA through neutrophil dysfunction.

One such experiment that would help strengthen this line of research would involve incubation of neutrophils with IgG after stimulation with PMA/LPS.

Through this, it would be possible to examine if neutrophils were capable of extracellularly modifying immunoglobulin G to the extent that had been observed from the mass spectrometry data.

The results found in this thesis pertaining to the effect of RA serum on neutrophil function provide preliminary data on the effect of individual RA patient sera with varying degrees of disease severity. Previous studies use pooled serum from RA patients, yet this data selected eight serum samples from RA patients to assess the response between various parameters, including cadmium. Further experiments would expand these experiments to include additional samples from each category, as each individual parameter effectively had a single sample to assess the neutrophil response. It would also be wise to repeat these primary neutrophil assays with two changes to the conditions. The first would expand this initial work through using higher serum concentrations to determine if RA serum has any effect on healthy primary neutrophils. Secondly, and more interestingly, RA patient neutrophils would be used in the assays throughout this thesis to see if RA patient autologous serum elicits any changes in general function.

The findings from the mass spectrometry highlight the strongest foundation for further RA research. These preliminary findings need to be explored further with even more samples being analysed via mass spectrometry. In addition to this, Biacore would be used to examine protein-protein interactions between the

patient serum antibodies and the modified Fc fragment in order to understand the binding kinetics that may explain the findings in **Section 5.3.3**.

Although modifications to alpha-1 antitrypsin were observed based on mass spectrometric data, this thesis did not investigate this further in terms of assessment of the proteomic response to A1AT. This work may prove even more relevant to the connection between RA and BR. As previously stated, A1AT deficiency is linked already to autoimmune diseases and bronchiectasis, though no work on patients that have both these conditions. Therefore, measurement of “Anti-A1AT” antibodies, be it through carbamylated or citrullinated variants of A1AT, may prove to be useful in predicting RA onset in BR patients. In addition to this, exploring the effects of citrullination and carbamylation on A1AT function may prove interesting to see if this protein may play a role in RA pathology.

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